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THE INHIBITION OF BACTERIAL LUMINESCENCE BY A HOMOLOGOUS SERIES OF CARBAMATES[1]

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FIVE FIGURES

The inhibition of bacterial luminescence by ethyl carbamate (urethane) and ethyl alcohol has been studied at some length and the parameters of drug concentration, temperature and hydrostatic pressure have been partially analyzed on a kinetic basis (Johnson, Eyring et al., '45). Both compounds cause a reversible inhibition that is increased by a rise in temperature and decreased by a rise in hydrostatic pressure. The quantitative data have indicated that they act by promoting a reversible protein denaturation of a catalyst, presumably luciferase, of the light-emitting oxidation, through an equilibrium change that takes place with a large volume increase and high heat and entropy of reaction. At high concentrations or temperatures, they catalyze an irreversible denaturation. The kinetic analyses have indicated also that more than a single equilibrium combination occurs between each of these drugs and the system affected, a phenomenon for which a parallel has recently been found in a study of the acceleration of the thermal denaturation of pure tobacco mosaic virus (TMV) by urethane at pressures of 1 and 680 atmospheres (Fraser, Johnson and Baker, '49).

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[1] This study has been aided in part by a grant from the American Cancer Society, through the Committee on Growth of the National Research Council, and in part by an Institutional grant from the New Jersey Section of the American Cancer Society to the Department of Biology for fundamental biological research. Some of the carbamates were kindly supply by the Eli Lilly and by the Ciba Pharmaceutical companies.

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The purpose of the present study has been to determine how the different members of a homologous series of urethanes act on bacterial luminescence. Representative members of the carbamate series from methyl to n-octyl, in moderate concentrations, were found in each case to cause inhibitions of luminescence, which were reversible by dilution or by centrifugation and resuspension of the cells in pure salt solutions. The present work deals essentially with these reversible effects only, in relation to drug concentration, temperature, and hydrostatic pressure.

METHODS

The species of luminous bacteria used in this study, *Photobacterium phosphoreum,* is a psychrophilic marine organism, growing best at 15 to 18°C., with a maximum brightness of luminescence at 22 to 23°C in suspensions of cells from agar cultures. Stock cultures were maintained on slants of nutrient agar containing 3% NaCl, 1% glycerol and 0.5% $CaCO_3$. For experiments, large plates or bottles of the same medium were surface inoculated and incubated for one day at 15°C., the cells then emulsified in 3% NaCl, washed twice with centrifugation and finally resuspended in a salt solution of 1.5% NaCl in 0.125 M Na_2HPO_4 — KH_2PO_4 at pH 7.0, plus 1% glucose. This suspension was aerated for 10 to 15 minutes and then placed in an ice bath as a source of material for several repeated experiments over a period of an hour or longer.[2] One volume of the stock suspension was added to 9 volumes of the inhibitor solution of desired concentration, made up in the corresponding buffered glucose salt solution, or to the solution alone as a control.

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[2] The experiments reported in this paper were carried out over a period of about two years during which there was considerable variation, for unknown causes, in constancy of luminescence intensity of control suspensions prepared in the same manner, as described. Generally, the light was constant for shorter times at higher temperatures, sometimes making comparisons between sucessive readings, that were taken over more than very short intervals of time, of doubtful meaning, and giving rise to irregular results. Although the purest reagents commercially obtainable were used, the presence of traces of poisons might have been a factor in these cases. The data described were obtained with suspensions whose luminescence remained adequately constant during the experiment.

The influence of different concentrations at constant temperature was determined by measuring the brightness of luminescence in a series of tubes prepared at the same time, and placed in a water bath, aerated by air from a manifold. Luminescence was recorded in repeated readings of each tube placed in front of a photocell with D.C. amplifier. The influence of temperature was similarly determined with control and inhibitor-containing suspensions, which were initially cooled to a low temperature and whose luminescence was recorded at small increments of temperature as the tubes were gradually warmed up, over a period of about 10 minutes, from a temperature well below the optimum up to about 35°C.

In experiments with hydrostatic pressure, a control or inhibitor-containing suspension was introduced into a pressure chamber, or "bomb," of the type described earlier (Johnson and Schlegel, '48), previously equilibrated at the desired temperature. The bomb was placed in a water bath and the intensity of luminescence was recorded by means of a photomultiplier, at normal and increased hydrostatic pressures up to 8,000 p.s.i

RESULTS

Influence of drug concentration

At 15°C. to 17°C. the brightness of luminescence in the washed cell suspensions remained in a steady state at approximately the same level for 20 to 40 minutes.[3] In figure 1, the averaged values from repeated readings were taken in plotting the relation between drug concentration and amount of inhibition. For most of the homologous series of carbamates represented in this figure, the difference between molar and molal concentrations is very slight. The data are therefore plotted as log $(\frac{I_1}{I_2} - 1)$ against log molal concentration of inhibitor added, essentially in accordance with the formulation derived earlier, where molar concentrations are used (Johnson, Eyring and Williams, '42; Johnson, Eyring et al., '45) and where I_1 represents the intensity of the control, and I_2 represents the intensity in the presence of a given inhibitor concentration. In most cases the relationship in linear, as would be expected provided the inhibitor acts on a single component, presumably protein, of the system. The slope of the line then indicates the average ratio of drug to protein molecules in the complex that is formed. For n-octyl carbamate the slope is 1.0, but there is a tendency for the slope to increase as the length of the aliphatic chain decreases, amounting to 1.6 with n-propyl carbamate. With the lowest members of the series, i.e., methyl and ethyl carbamate, the slope for inhibitions of less than 50% is approximately two, but there is a decided increase in slope as the inhibition becomes greater with higher concentrations of the drug. The facts that some of the slopes have numerical values

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[3] See footnote 2 on page 2.

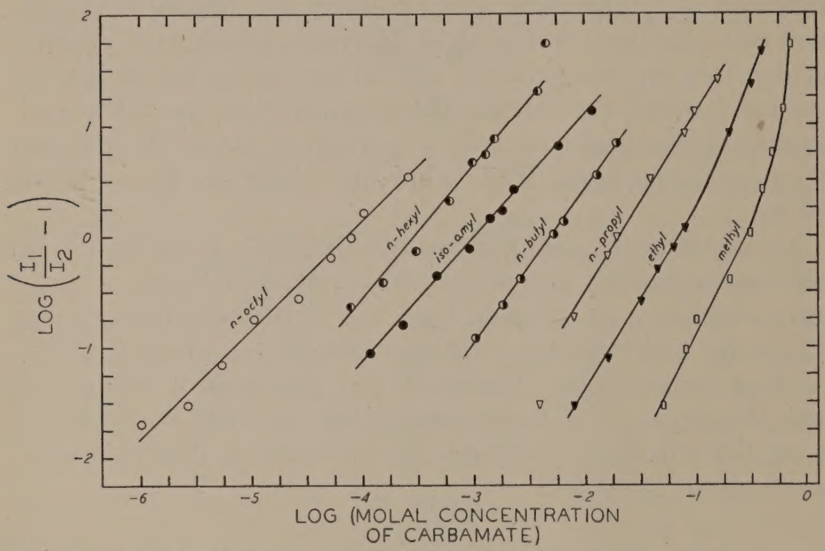

Fig. 1 Relation between concentration and inhibition of luminescence in *Photobacterium phosphoreum* by different carbamates. Data plotted as described in the text.

that are not integers, and that some of the slopes change at high concentrations of the inhibitor, indicate that multiple equilibria are concerned. Further evidence that this is the case is apparent from the data with respect to hydrostatic pressure, discussed later.

The relation between thermodynamic activity and potency of various narcotics has been discussed by Brink and Posternak ('39). They found that the log mol fractions of a homologous series of alcohols required "to just suppress" light emission in *Achromobacter fischeri,* according to the data of Taylor ('34), was directly proportional to the log of the activity coefficient of the alcohol (Butler, '37) from methyl to n-octyl. Although the physical data with respect to the corresponding carbamates are lacking, on the very reasonable assumption that their coefficients increase with additional methylene groups by very close to the same ratio as the alcohols, a similar linear relationship was found between the log of the activity coefficient and the log of the mol fraction of methyl to iso-amyl carbamates.

When the data obtained in the present study (fig. 1) are plotted in accordance with equation (6) of Brink and Posterternak ('39), for inhibitions of 9, 50 and 91% respectively, using the same values for activity coefficients, the curves shown in figure 2 result. The relationship is evidently linear for the series of methyl to butyl, and possibly isoamyl carbamate, the slopes of the lines changing somewhat with the extent of inhibition: 1.35 for 9%, 1.05 for 50% and 0.9 for 91% inhibitions respectively. The points for n-hexyl and n-octyl carbamates all depart from the linear relationship that obtains with the lower members of the series.

The relationships shown in figure 1 are altered by a rise in temperature, e.g., at 25°C., some of the slopes are different, and the position of the lines with respect to the abscissae change according to whether the potency is greater or less at this temperature. The influence of a wide range of temperatures on the inhibition caused by single concentrations of representative carbamates is illustrated in figure

3. Thus, with methyl carbamate, the inhibition increases at the higher temperatures, somewhat as shown previously for ethyl carbamate (Johnson, Eyring and Williams, '42; Johnson, Eyring et al., '45), while the opposite is true for propyl and higher members of the series. Moreover, with moderate concentrations of the latter, at temperatures above the normal optimum, the brightness of luminescence is actually somewhat greater than that of the control, even though at temperatures below the optimum the same concentrations cause inhibitions of as much as 60 to 85%. The "stimulation" at high temperatures may amount to as much as double that of the control. This effect must take place through a different mechanism than that of inhibition at low temperatures.

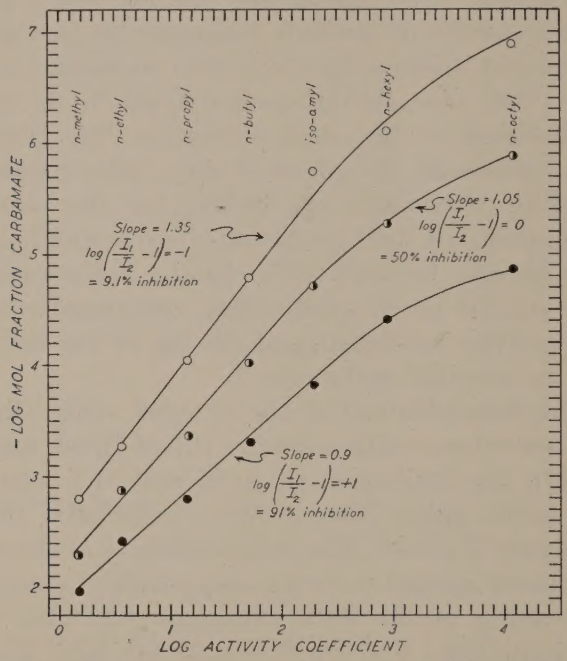

Fig. 2 Relation between thermodynamic activity coefficients of different carbamates and the partial mol fractions required for three respective extents of inhibition of luminescence intensity in *P. phosphoreum*.

The temperature effects illustrated in figure 3 are in contrast to what would be expected if all members of the series acted by precisely the same mechanism. Further differences in their respective actions are demonstrated by the influence of pressure.

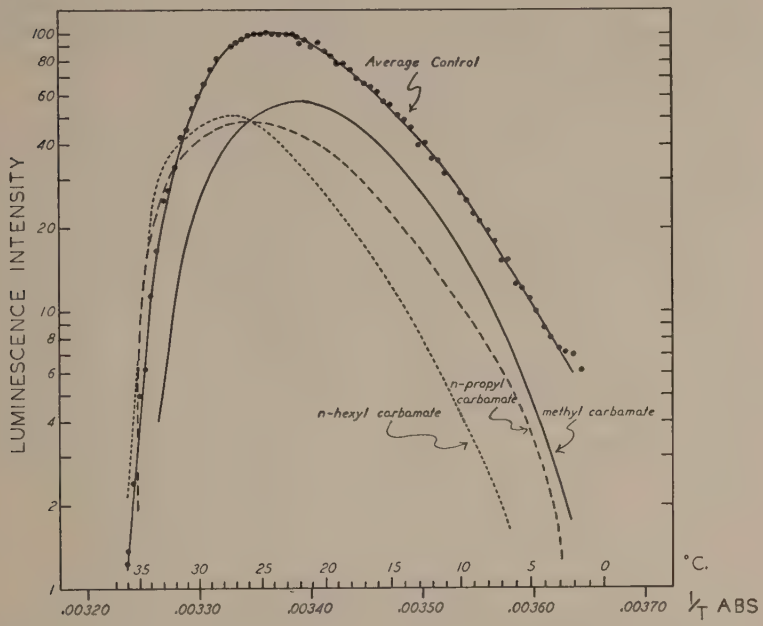

Fig. 3 Influence of temperature on the effects of methyl, n-propyl, and n-hexyl carbamates, respectively, on luminescence intensity. Maximum intensity of the control arbitrarily taken as 100%. Points on the control curve are individual or averaged points from 19 repeated experiments; points on the other curves are omitted from the figure to avoid crowding.

Moderate pressures cause a reduction in the amount of inhibition caused by various concentrations of methyl carbamate. The data are plotted in figure 4 in accordance with the formulations referred to above. The initially straight line portions of the curves relating the logarithm of inhibition $(I_1/I_2 - 1)$, to hydrostatic pressure indicate that the pressure effect in this region is mediated for the most part through a single equilibrium reaction, which has net volume increase of between 60 and 70 cm^3 per mole in the final over the initial state. At higher pressures, however, the curves change markedly in slope, especially with the lowest drug concentrations, indicating that a different equilibrium reaction is largely limiting in this region of pressures. The different reaction, however, may involve exactly the same reactants, i.e., it may involve the same enzyme.

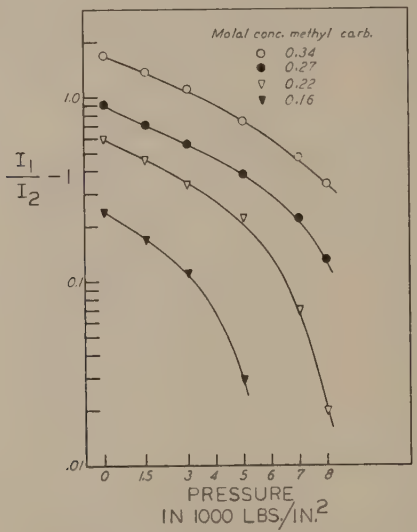

Fig. 4 **Influence of hydrostatic pressure on the inhibition of luminescence by different concentrations of methyl carbamate at 17°C.**

With different members of the carbamate series, in concentrations appropriate to bring about an inhibition of approximately 50% in each case, the influence of pressure is to reduce the amount of inhibition caused by the lower members (methyl, or ethyl), but to increase the amount caused by the higher members (n-hexyl, n-octyl) as shown by the data plotted in figure 5. The action of intermediate members (n-propyl, n-butyl, or iso-amyl) shows little sensitivity to pressure under these conditions. The data for n-amyl carbamate, not included in the figure, were very similar to those for iso-amyl.

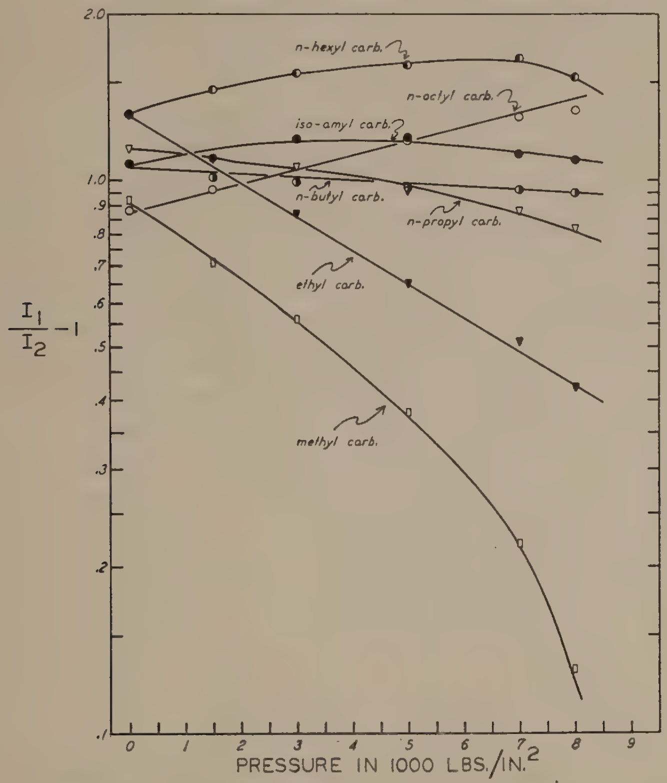

Fig. 5 Influence of hydrostatic pressure on the inhibition of luminescence by various carbamates in concentrations required to cause about 50% inhibition at normal pressure and 17°C.

DISCUSSION

The pronounced differences in the influence of temperature and of pressure on reversible inhibitions caused by the several carbamates included in this study show that, even among closely related members of a homologous series, the equilibria which they establish with their site of action may be of very different types. Although it has not been demonstrated that each of the members acts directly on the light emitting system, by studies with partially purified systems *in vitro,* e. g., that of *Cypridina* (cf. Harvey, '40), the known sensitivity of luminescence to narcotics in general makes it seem very likely that they do. Assuming that this is the case, the question remains as to the reason for the apparent differences in action. Although the intimate mechanism cannot be described with assurance on the basis of the data at hand, the following considerations are apropos.

The quantitative relationships discussed earlier with respect to the action of urethane and ethyl alcohol on luminescence indicate that each of these drugs establishes more than one equilibrium with the light emitting enzyme system (Johnson, Eyring et al., '45). Although the general nature of these reactions appeared to be that of promoting, or catalyzing, a reversible, and under some conditions (high concentrations or temperatures) irreversible denaturation of the catalyst, they were not all characterized by the same ratio of drug to enzyme molecules in the combination concerned. Consequently, the observed effect of temperature, pressure, or concentration depended on which one of the several reactions was predominant at the various magnitudes of these variables. More recently, a kinetic analysis of the influence of urethane on the rate of thermal denaturation of pure tobacco mosaic virus has provided evidence of a corresponding situation with an *in vitro* system, to the extent that small concentrations of this drug catalyze the denaturation of the protein through several independent reactions with different heats, volume changes and numbers of drug molecules combined per protein molecule in the activated complex (Fraser, Johnson and Baker, '49). There appears to be no doubt, therefore, that drugs of this type are capable of entering into several independent, simultaneous reactions with a single protein. Moreover, there is no *a priori* reason to expect that these different reactions should have similar thermodynamic or rate constants of heat, volume change, etc.

On these grounds, it seems more readily understandable that variations such as those described would be encountered among a homologous series of compounds acting on the same system. Presumably each one should be capable of entering into more than one reaction with the same system, while the properties of the specific drug would determine the particular reaction that would predominate under given conditions. In the present instance, there are indications that, in general, similar effects are caused by high concentrations of the lower members of the series as low concentrations of the higher members. Investigation of high concentrations of the higher members are limited both by their low solubility in the aqueous phase, and by the potency of their effects in low concentration, with the consequence that the additional, presumably possible types of action cannot be readily observed.

In a more specific sense, it is apparent that the temperature and pressure relations of methyl or ethyl carbamate inhibitions are closely related to the mechanism of reversible protein denaturation, because of the evidently large volume changes and heats of reaction. In the previous studies, this type of action has been referred to as "type II." On the other hand, the chief effects of the higher carbamates are similar, in their pressure and temperature relations, to inhibitions that take place independently of the denaturation equilibrium, thereby resembling the kind referred to earlier as "type I," and exemplified by the action of sulfanilamide on luminescence. The simplest interpretation of the seemingly anomalous behavior of the higher carbamates is that they inactivate the enzyme primarily by combining at the surface of the protein, rather than primarily with hydrophobic groups normally present within the interior and which become available through an unfolding process accompanied by a considerable volume increase, as in the denaturation reaction.

Whatever the intimate mechanisms, it is apparent that a number of equilibrium reactions with different characteristics are possible not only between a single member of the homologous series and the luminescent system, but also between the different members of the series and this system.

SUMMARY

At 15°C. and atmospheric pressure, the concentrations of respective members of a homologous series of carbamates, from methyl to iso-amyl, required to produce a given inhibition of luminescence in *Photobacterium phosphoreum* are proportional to their thermodynamic activity coefficients, but n-hexyl and n-octyl carbamates deviate from this relationship.

Analysis of the relation between drug concentration and amount of inhibition indicates that the average ratio of drug to enzyme molecules increases from unity for octyl carbamate to two or more for methyl carbamate.

With rise in temperature, at normal pressure, the amount of inhibition by methyl or ethyl carbamate increases, while that caused by propyl or higher members decreases. With the latter, at temperatures above the normal optimum of luminescence, the inhibition caused by a given concentration at the lower temperatures is replaced by a ''stimulation'' amounting to double the intensity of the control.

At 25°C., with concentrations of methyl or ethyl carbamate causing approximately 50% inhibition at atmospheric pressure, the amount of inhibition decreases with hydrostatic pressures up to 8,000 p.s.i. With corresponding inhibitions by n-propyl, n-butyl and n-amyl or isoamyl carbamates, the effect of pressure is successively less, while with n-hexyl and n-octyl carbamates it is increased by pressure.

The results indicate that a single member of the carbamate series may enter into several equilibrium combinations with the luminescent system, and that different members may form predominately one or another type of reaction with this system.

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ACTIVITY OF NARCOTIZED AMPHIBIAN LARVAE UNDER HYDROSTATIC PRESSURE[1]

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Princeton, New Jersey

TWO FIGURES

The modification, by hydrostatic pressure, of narcosis in tadpoles has been reported in a recent preliminary note (Johnson and Flagler, '50). The possibility of such a relationship was anticipated by experiments with luminous bacteria where it was found that moderate inhibitions of luminescence by certain drugs, e.g., urethane, ethyl alcohol, and others, could be immediately and almost completely abolished by raising the hydrostatic pressure from atmospheric to about 5,000 p.s.i. (Johnson, Brown and Marsland, '42a, b; Johnson, Eyring, Steblay, Chaplin, Huber and Gherardi, '45). In the present report, further observations with larvae of *Amblystoma maculatum,* as well as with frog tadpoles, are described, together with some remarks about the experimental method and possible significance of the results.

METHODS

A diagram of the two essential parts of the steel pressure chamber, or "bomb," having an outer diameter of 5 inches, an inner diameter of 3½ inches, and at opposite ends two herculite plate glass windows 2 inches in diameter by 1 inch

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[1] This study has been aided in part by a grant from the American Cancer Society, through the Committee on Growth of the National Research Council, and in part by an Institutional grant, from the New Jersey Section of the American Cancer Society to the Department of Biology, for fundamental biological research.

thick, has been given in a paper by Botts, Morales and Johnson ('50).

In the present study, a thin walled lucite cylinder, 2 inches in diameter, was mounted on a brass disc which could be screwed against the vertical wall at one end of the bomb, in order to furnish a "cage" that would keep the larvae in line of view between the two windows (fig. 1). A number of small perforations were drilled through the lucite to allow diffusion and convection of the water between the cage and larger chamber of the bomb, thereby affording a greater total supply of dissolved oxygen for respiration during the period when the bomb was closed and additional oxygen could not be readily supplied from the outside.

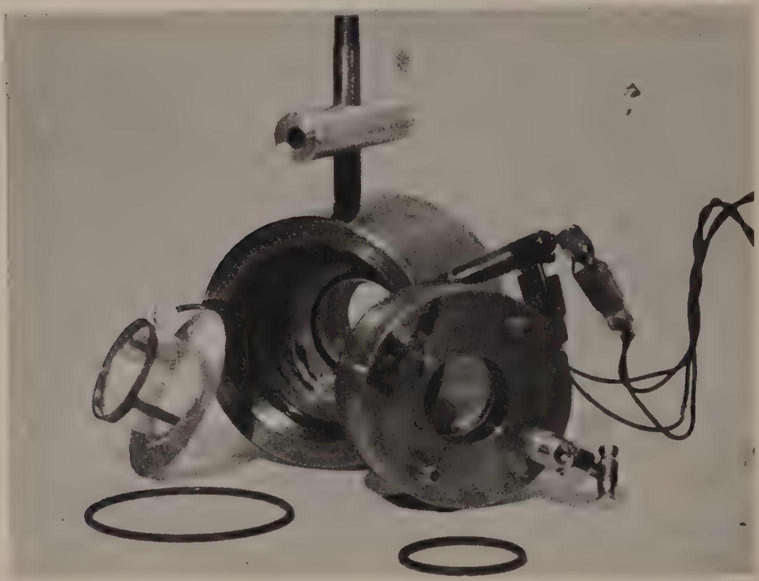

Fig. 1. The pressure bomb, as described in the text, showing the removeable lucite "cage" at the left, and threaded male part at the right. In the foreground are "O" washers, the larger of which is used to seal the threaded joint between the two parts of the bomb. Washers of the smaller type seal the windows against leakage under pressure.

To provide satisfactory illumination for photographic purposes, two ordinary 3.8 volt flash light globes were mounted inside the pressure chamber above the lucite cage. One of the connections to the globes was grounded to the bomb, and the circuit was completed to the other by means of an insulated wire, soldered to a small, solid brass disc on the end of a brass rod communicating with the outside through a hollow shaft of a steel rod screwed into the wall of the bomb. A thin layer of bakelite insulated the disc and brass rod, while a small rubber washer beneath the disc sealed against loss of pressure by leakage. With this arrangement the illumination was not quite adequate for taking motion pictures but was sufficient for exposures of 0.25 second to 0.1 second at f.4.5, on super XX film.

Young tadpoles of *Rana silvatica,* measuring between 1.5 and 2.0 cm in total length, were obtained from several sources in neighboring ponds. The *Amblystoma* larvae developed from eggs brought into the laboratory about 5 weeks previously. For observations with unnarcotized animals, the bomb was turned on its side, filled with tap water, and several larvae introduced into the cage, along with one or more glass beads as a means of testing for response to mechanical stimulation, by tilting of the bomb. The bomb was then closed, righted, and attached to the oil line from a hydraulic pump for application of pressure. In experiments with narcotized animals, the larvae were first placed in an open vessel containing the desired drug concentration in tap water until spontaneous activity and response to gentle prodding ceased. They were then transferred to the bomb (filled with the same solution) and a similar procedure followed as with the controls. The animals very usually kept within the closed bomb for a maximum of about 15 minutes, in order that the supply of dissolved oxygen would not be seriously depleted. The total period under pressure was usually not more than 2 to 5 minutes, which was sufficient for observation of the effect, since the pressure could be quickly applied and its influence was readily manifested.

RESULTS

Unnarcotized animals

With unnarcotized tadpoles, the application of pressures of between 1,500 and 2,500 p.s.i. caused a pronounced increase in swimming activity. The *Amblystoma* appeared to be somewhat more sensitive to pressure, showing increased activity at about 1,000 p.s.i. These pressures, maintained for short periods of a minute or two, seemed to cause no injury. As the pressure was increased to 4,000 or 5,000 p.s.i., however, activity decreased and the apparently normal swimming movements were replaced by irregular twitches, fibrillations, or spasms, and the tails became rigidly bent. After release of these higher pressures, the animals remained motionless with bent tails, for periods up to several hours, depending on how long they had been under pressure, but they eventually recovered. The *Amblystoma* usually recovered faster than the frog tadpoles.

Alcohol narcotized larvae

In concentrations of 2.5 to 6.0% alcohol, at 22 to 26°C., spontaneous motility and response to gentle mechanical stimuli ceased within about 5 to 20 minutes. The stronger concentrations required shorter times, as would be expected, and the response to increased pressures was less pronounced. Most of the experiments were done with minimal concentrations needed for complete inactivity at normal pressure.

With a gradual rise in pressure, tadpoles usually became active at 2,000 to 3,000 p.s.i., and moved about in an apparently normal manner. With some groups of individuals, higher pressures were needed, and the swimming activity was soon followed by spasms. The animals then settled to the bottom. Pressures of 4,500 to 5,000 p.s.i., gave rise to signs of injury similar to those observed with unnarcotized animals, but they were always preceded by at least a brief restoration of swimming activity. Only one group of individuals, out of many that were studied, failed to evidence recovery from narcosis. Unlike the others, this group had hatched in the laboratory from eggs that had been refrigerated for some time; a possible factor in the atypical response. After release of high pressures (5,000 p.s.i.) recovery was slower than with unnarcotized animals, and the majority usually died within a day or two. With *Amblystoma,* recovery was much better, and only a small proportion died subsequently. Moreover, lower pressures, in general, were needed to bring about activity. The swimming of either narcotized or unnarcotized *Amblystoma* was characterized by the somewhat clonic movements ordinarily observed with the normal larvae. With either frog tadpoles or *Amblystoma,* repeated swimming activity followed by inactivity took place as the pressure was raised to about 2,500 p.s.i., and then released (fig. 2).

Urethane narcotized tadpoles

Experiments with urethanes showed that the influence of pressure on narcotized animals depends on the specific narcotic, even in a homologous series. In ordinary urethane (ethyl carbamate) at a concentration of 0.08 to 0.1 M., the results were similar to those in 3% alcohol. Activity was readily restored as the pressure was raised to around 2,500 p.s.i.

Under an approximately minimal narcotizing concentration of about 0.001 M. n-amyl carbamate, compression failed to bring about recovery of activity. At most, a few feeble twitches occurred in some of the individuals, and the higher pressures, 4,500 to 5,000 p.s.i., resulted in evidences of injury similar to those that took place in the other cases.

Experiments were undertaken also with sodium barbital, but were abandoned because of peculiarities in the effects of the drug. Saturated solutions, at slightly alkaline pH, did not cause narcosis over considerable period of time. When transferred back to tap water, evidence of narcosis became apparent.

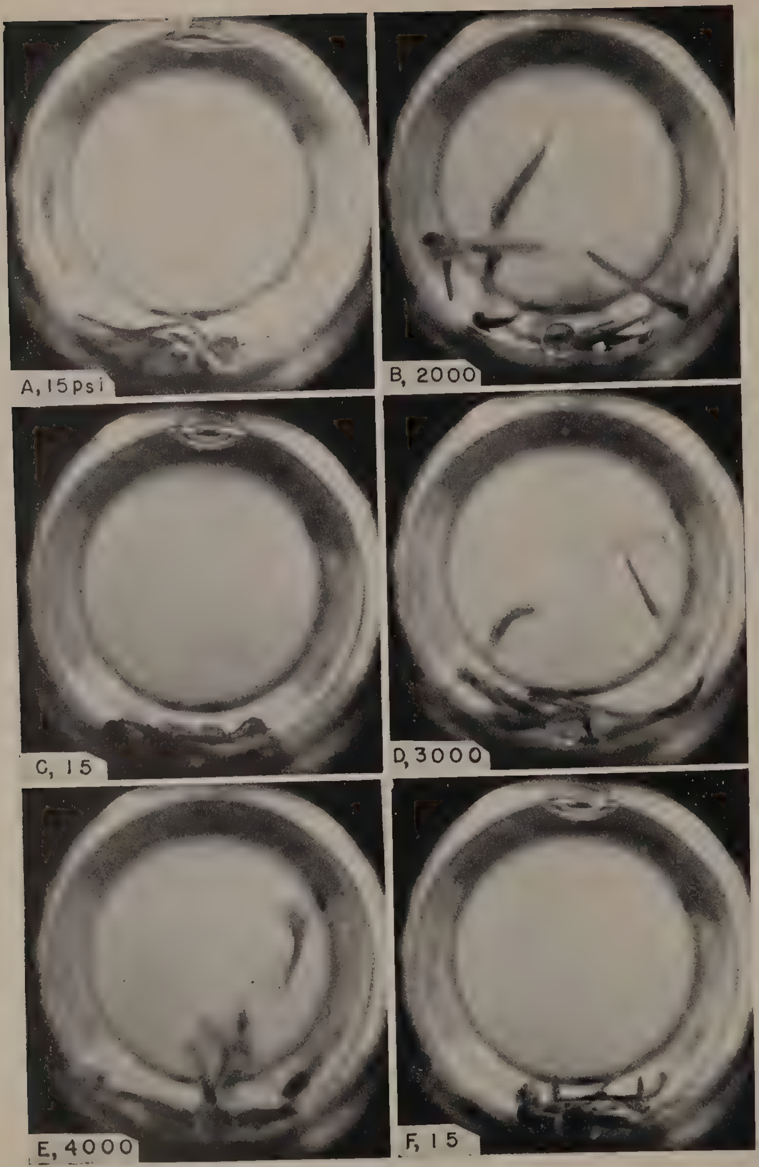

Fig. 2 Activity of *Amblystoma* larvae, in 2.5% alcohol, with successive changes in hydrostatic pressure, over a total period of 4 minutes. Photographs were made through one of the windows of the bomb. Note that air bubble, at top of cage disappears under pressure, reappears after release of pressure. The slight increase in amount of dissolved oxygen under pressure that occurred in this manner, cannot be responsible for the decreased narcosis, inasmuch as similarly narcotized animals showed no recovery when transferred to a corresponding narcotic solution that had been equilibrated with pure oxygen.

DISCUSSION

Because of its bearing on the general theories of narcosis and anesthesia, the most important aspect of the foregoing observations, from the point of view of the present study, is the action of pressure in bringing about activity of narcotized animals. Two other aspects, however, call for brief mention and will be considered first, namely, the increase in activity of unnarcotized animals under moderate pressures, around 2,500 p.s.i. and the injurious effects of higher pressures, around 4,000 to 5,000 p.s.i. These various effects evidently derive from different, though perhaps related, mechanisms.

At appropriate temperatures, moderate increases in pressure have been shown to augment a number of biological processes, both in isolated tissues, e.g., the rhymthmic contraction of heart muscle (cf, review by Cattel, '36) and in whole organisms, e.g., the activity of marine animals (Ebbecke, '35) or the intensity of bacterial luminescence (reviewed by Johnson, '47; Johnson and Eyring, '48). At higher pressures, the same processes are retarded. A basic mechanism which accounts, in simplest terms, for this general type of pressure effect has been worked out in connection with luminescence. It is based on the evidence that enzyme systems, and to some extent other proteins, may undergo reversible as well as irreversible denaturation, accompanied by large molecular volume increases of reaction, or of activation, respectively, with the result that these reactions can be reversed or retarded by pressure. At temperatures near the normal optimum, a large enough fraction of a critically important enzyme system may exist in a partially denatured state for the net rate of the process to increase under pressure, because of the influence of pressure in shifting the equilibrium towards the undenatured, active form of the protein, thereby effectively increasing the amount of active catalyst. Under still higher pressures, however, the catalytic reaction itself, which may also proceed with a considerable volume increase, is sufficiently opposed by pressure to retard the over all rate. These two chief reactions, equilibrium and rate process, assume limiting rôles at different temperatures and pressures, as determined by the numerical values of the constants for heat and volume changes in the specific reactions. In luminescence, a single system appears largely to limit the total rate, and the two reactions suffice to account quantitatively for the chief effects of various temperatures and pressures.

Although the readily reversible inhibitory effects of higher pressures may be accounted for by the above interpretation, the injurious and more or less permanent effects could arise from a variety of causes. Further discussion of the latter would not be justified here.

With specific reference to the problem of narcosis, the correct interpretation hinges upon the question as to whether (1), a true reversal of narcosis was effected by pressure, in the sense that the drug was dissociated from its critical site of action, or (2), the activity of the narcotized animals was brought about *solely* by a direct action of pressure on muscle or nerve through mechanisms not influenced by the presence of narcotics. On the grounds that the movements of narcotized larvae under moderate pressures often appeared perfectly normal and coordinated, the latter possibility does not seem altogether likely. On the other hand, pressure is known to act directly on the contractile mechanism of muscle (Brown, '34, '34–35, '36) and to cause twitches or contractures of isolated muscle, even after it has been rendered unresponsive to ordinary stimuli by treatment with curare, alcohol, or sucrose (Ebbecke, '14). Moreover, Ebbecke ('36) has reported rhythmic, clonic movements of the extremities of a spinal frog under pressure. Direct effects, apart from the presence of narcotics, are, indeed, very likely responsible to some extent for the results observed with higher pressures, and they do not exclude the first of the above-mentioned possibilities.

In support of the view that the primary action of moderate pressures actually consisted in a reversal of narcosis are some remarkable parallels between the action of ethyl alcohol, urethane and n-amyl carbamate on the activity of the amphibian larvae and on the intensity of bacterial luminescence, where the analyses have been much more complete and convincing. Thus, in both cases, (1) the respective concentrations required for inhibitions that are readily reversible by removal of the drug are approximately the same, (2), pressure reversibly opposes the action of the first two, while scarcely affecting that of the third, (3), the effect of pressure is manifested almost immediately, and (4), the magnitude of the pressures required is the same (Johnson et al., '45; '50). To these it may be added that the minimal narcotic concentration of ethyl alcohol for amphibia (frogs) is markedly less at 30° than 3°C. (Meyer, '01); the same is true in luminescence.

The simplest interpretation for the recovery of activity in narcotized animals under pressure is according to the same mechanism as the one involved in luminescence. It is only necessary to postulate that the narcosis occurs chiefly through the reversible inhibition of a critical system, e.g. a rate limiting enzyme of the central nervous system, by similar equilibrium reactions. This view is consistent with the now generally accepted theory that the action of narcotics is mediated primarily through enzyme inhibition. The observations are difficult to harmonize with some older, but still classic theories (cf. review by Henderson, '30), for example, that of lipid solubility, inasmuch as purely hydrostatic pressure, of the magnitude employed in this study, is known to have very little effect on solubility.

Unfortunately, the quantitative effects of drugs in relation to concentration, pressure and temperature cannot be so readily and accurately measured with whole animals as with the simpler process of luminescence. Evidence that fundamentally the same mechanism is involved, in both cases, at the molecular level, adds interest and significance to results obtained with the simpler system. The general parallels that have been pointed out lend support to the view that the basic mechanism is, indeed, very similar.

SUMMARY

Unnarcotized larvae of *Rana silvatica* and *Amblystoma maculatum* at room temperature, 22–26°C., show increased spontaneous activity during brief periods of moderate hydrostatic compression (ca., 2,500 p.s.i.) and cessation of activity, with evidence of injury, after brief compression at 4,500 to 5,000 p.s.i. Gradual recovery subsequently took place at normal pressure.

In 2.5 to 6.0% ethyl alcohol, or in 0.08 to 1.0 M. ethyl carbamate, which cause loss of spontaneous activity as well as response to gentle mechanical stimuli, the application usually of moderate pressures restores spontaneous activity and apparently normal swimming movements. Under high pressures activity again ceases, with evidence of injury. Although gradual recovery took place subsequently in narcotic free tap water at normal pressure, the frog tadpoles generally died within a day or two. *Amblystoma* larvae were more sensitive to pressure, recovered sooner subsequently, and usually survived.

When narcotized in 0.001 M. n-amyl carbamate, there was scarcely any recovery of activity under increased hydrostatic pressure.

These results with amphibian larvae have a consistent parallel with respect to the action of pressure on the inhibition of bacterial luminescence by the same agents in corresponding concentrations, and their possible significance in the general theory of narcosis is briefly discussed.

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THE ELASTIC MECHANISM AND HYDROGEN BONDING IN ACTOMYOSIN THREADS * ‡

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ELEVEN FIGURES

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INTRODUCTION

This paper reports thermoelastic experiments on actomyosin threads, undertaken with a view to studying the molecular nature of the tension in such threads and, by analogy, in muscle fibers. The investigation entailed as well some preliminary study of the nature of the molecular cross-linkages existing in the threads — cross-linkages which, as will be seen below, seem to be in part H-bonds.

Although the chief interest in thermoelastic experiments lies in the structural interpretations which can be given to them (see, for instance, Guth, '48; King and Lawton, '50), the actual experimental equations are largely thermodynamic. For this reason it will be convenient at the outset to summarize this type of analytic treatment, together with some representative applications made in the past.

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* The opinions or assertions contained herein are the private ones of the writers and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large.

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We shall take as the system a fiber of length L, at equilibrium tension, τ, immersed in a fluid.[2] The combined volume of fiber and fluid is V, and the uniform pressure and temperature throughout the system are, respectively, p and T. The basis for these studies is the equation,

$$\tau = (\partial H/\partial L)_{p,T} - T(\partial S/\partial L)_{p,T} \tag{1}$$

where H and S are, respectively, the enthalpy and the entropy of the system. A negative value of $(\partial S/\partial L)_{p,T}$ (and therefore a positive entropic contribution to the tension) is interpreted statistically as the configurational entropy decrease resulting from the unkinking of long chain molecules. The entropic contribution to the tension, $-T(\partial S/\partial L)_{p,T}$, can be expressed in various ways. At constant pressure, a positive contribution is indicated by:

(2) An isometric increase in tension with increasing temperature. The contribution is $T(\partial \tau/\partial T)_{p,L}$.

(3) An isotonic decrease in fiber length with increasing temperature. The contribution is $- T(\partial \tau/\partial L)_{p,T}\ (\partial L/\partial T)_{p,\tau}$.

(4) A rise in temperature with adiabatic (or rapid) stretch. The contribution is $C_{p,L}\ (\partial T/\partial L)_{p,S}$ where $C_{p,L}$ is the heat capacity at constant pressure and constant fiber length.

(5) A loss of heat with isothermal stretch. The contribution is . . . $-(\delta q_{rev}/\partial L)_{p,T}$, where δq_{rev} is the reversible heat absorbed.

Much interest attaches not only to the absolute value of the "entropic tension," $-T(\partial S/\partial L)_{p,T}$, but also to its importance relative to the "enthalpic tension," $(\partial H/\partial L)_{p,T}$. Since τ can be measured independently of $(\partial S/\partial L)_{p,T}$, the enthalpic contribution can be obtained by simple transposition of equation (1). For many fibers, of which lightly vulcanized rubber is representative, $(\partial H/\partial L)_{p,T}$ relative to τ is small up to moderate extensions, and, in accordance with statistical-mechanical theory, it is concluded that the elastic mechanism of such systems is largely entropic, i.e., due to the "Brownian pull" of unkinked long chains. For rubber at high extensions, and for a second class of natural fibers, $(\partial H/\partial L)_{p,T}$ is found to be large relative to τ (and the entropic mechanism is correspondingly unimportant). A mechanical interpretation of $(\partial H/\partial L)_{p,T}$ is tenuous in the absence of information about the importance of $p(\partial V/\partial L)_{p,T}$ relative to $(\partial E/\partial L)_{p,T}$, where E is the internal energy.[3] It is ordinarily assumed that $p(\partial V/\partial L)_{p,T}$ is negligible, and therefore that,

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[2] The extension of the system to include the solvent is necessary for the proper introduction of the pressure parameter; it also corresponds more closely to the usual biological situation. The treatment of systems in which the composition of the solvent may vary is also of physiological importance, and is being developed by Dr. T. L. Hill ('50).

$$(\partial H/\partial L)_{p,T} \cong (\partial E/\partial L)_{p,T}. \qquad (6)$$

If (6) is assumed, a positive "enthalpic tension" is usually attributed to the elastic deformation of valence bond distances or angles as a result of stretch. Especially in the case of protein fibers immersed in water, electrostatic interactions between charges fixed on the chain molecules (e.g., charges arising from ionization) can also contribute to E, and, insofar as these interactions depend on macroscopic length, to $(\partial E/\partial L)_{p,T}$. Thus an enthalpic tension either positive or negative can in certain cases be attributed to an electrostatic contribution of the type just mentioned.

Hill and Hartree ('20) found that resting muscle warmed during quick stretch. Feng ('32) confirmed this finding for moderate extensions, and noted further that the reverse was true at high extensions.[4] These findings, interpreted through (4), show that for moderate extensions the entropy makes a positive contribution to the tension, and for high extensions a negative one. This general result (there persist differences of opinion regarding the existence of inversions points at very low and very high extensions)[5] has been more firmly established by Wöhlisch ('40) using the method of (3), and by Meyer and Picken ('37) and Buchthal ('44) using the method of equation (2). It has been shown, moreover, that the entropic tension makes up most of the tension at moderate extensions. However, the Ramsey-Street ('40) observation that the resting tension of a muscle fiber is largely due to the sarcolemma makes it doubtful whether any of the foregoing conclusions apply to the resting contractile mechanism itself. The metabolic (and presumably thermal) response to stretch discovered by Feng ('32) is also likely to be a complicating factor in most of the same experiments.

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[3] We are currently investigating this question for actomyosin threads, evaluating $p(\partial V/\partial L)_{p,T}$ by means of the equivalent coefficient, $-p(\partial\tau/\partial L)_{p,T}\,(\partial L/\partial p)_{\tau T}$. In some instances the system is such that it can be studied at $p = 0$, but this is rarely ever the case for biologically interesting fibers, which are ordinarily studied at atmospheric pressure.

[4] In developing a formula for calculating a linear coefficient of thermal expansion, Hill and Hartree ('20) make some explicit mechanical assumptions, and also the implicit assumption that equation (5) for the isothermal process is a good enough approximation to the adiabatic situation which they studied experimentally. Feng ('32) employed the same equation.

The thermal complications imposed by active metabolism, and the additional complications resulting from non-equilibrium situations, have discouraged thermoelastic analyses of excited muscle. Bull ('46), in the development of his contraction theory, reports positive vaues of $(\partial\tau/\partial T)_{p,L}$, and therefore [by (2)] a positive entropic tension for tetanized single muscle fibers. He has also fitted statistical equations (assuming a purely entropic tension) tolerably well to the $\tau(L)$ curve of fibers contracting in the δ-state (Ramsey and Street, '40). The uncertainties in these analyses are fully discussed by Bull; it seems fair to say that this work is valuable and suggestive, but cannot yet be regarded as conclusive.

The difficulty of analyzing the tension in muscle fibers suggests the study of actomyosin films and fibers, which in a certain measure can be regarded as models of muscle and apart from this are interesting objects of study. The only[6] such investigation seems to be that of Woods ('46) on oriented films, discussed in relation to contraction theories by Astbury ('47) and by Sandow ('49). In Woods' films the tension analyzed by equation (2), was found to be almost entirely enthalpic, and the entropic contribution was negative. We shall show later that this is one of two opposite results obtainable with actomyosin, and therefore not entirely decisive in elucidating the nature of muscle tension.

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[5] Meyer and Picken ('37) reported a first inversion point at about 10% extension; this was disputed by Wöhlisch ('40) on the basis of measurements on "fascia-free" muscle. The existence of the second inversion has been doubted by Buchthal et al. ('44). Recently, Meyer and Hasselbach ('49) have suggested that the negative entropic tension sometimes observed at small extensions of protein fibers is due to the increase in the volume available for the chain segments to rattle in. While this molecular picture may be correct, it is irrelevant to the thermodynamic analysis. If a volume effect occurs, it will be properly included in the enthalpic term not the entropic; in any case $-(\partial\tau/\partial T)_{p,L}$ measures $(\partial S/\partial L)_{p,T}$, and no split of the latter term is justifiable on thermodynamic grounds.

In the present work we propose the actomyosin thread as a model for the contractile substance in unexcited muscle. Specifically, the similarity deemed to exist between the two systems is in thermoelastic behavior, and obviously not in all properties. There is little doubt that actomyosin (Szent-Györgyi, '47) is the predominant and the chief contractile protein in skeletal muscle. Moreover, the electron microscopic work of Hall et al. ('46) has shown that the actomyosin filaments within the myofibril are intrinsically contractile, and that these same filaments, fragmented transversely but otherwise undamaged, constitute extracted actomyosin. Extrusion and other procedures involved in the preparation of threads achieve a realignment of filaments (as judged by birefringence measurements; see Fischer, '36) comparable to that in muscle fibers, and simultaneously induce enough re-''vulcanization'' to knit the filaments into a three dimensional macroscopic network capable of sustaining tension. Finally, many investigators have recorded, as a result of the *in vitro* adenosine triphosphate-actomyosin interaction, a number of phenomena (e.g., enzymatic activity, viscosity response, etc.) which can be regarded as legitimate counterparts of processes occurring in the contraction-relaxation cycle. The stability of these properties as a function of time after the extraction of actomyosin is very high; for instance, in the present work ATPase activity is retained undiminished for periods up to two months. No doubt the most significant argument against the idea of using the reaction of ATP with a thread as a model of contraction is the failure of such a system to contract with a load (see, for instance, Barer, '48 and Sandow, '49). This is indeed an objection to employing a thread in the model of the *contraction* process — not because the fundamental reactions are necessarily different in the model, but because the mechanical arrangements are probably different, i.e., on reaction with the nucleotide the actomyosin elements coil (Jordan and Oster, '48) so violently against the load, that in the absence of sufficiently strong cross-binding there is a shredding of the network and a consequent extension. This objection is not, however, to be mistaken for an objection to the thread as a model for investigating the nature of the elastic mechanism of the actomyosin *prior* to the addition of ATP; with slowly applied small forces and in the absence of reagents capable of disrupting the cross-linkages, the mechanical network is not

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[6] Knappeis' ('48) observation of the fall in birefringence of threads when heated at constant length, and the fitting (King and Lawton, '50) of statistical equations (assuming a purely entropic tension) to the $\tau(L)$ curves of threads (Dubuisson and Monnier, '43) may be taken to suggest the importance of an entropic contribution, but are not conclusive in this respect; indeed results below make doubtful any conclusions based on the ability to fit statistical equations to these $\tau(L)$ data. In contrast to the situation with respect to actomyosin, related protein fibers have been very thoroughly analyzed. Wöhlisch et al. ('43) and Meyer and Ferri ('36), using the method of equation (2) have shown that the tension of elastic tissue (cervical ligament) is almost entirely entropic up to high extensions. Wöhlisch and Clamann ('32) reached the same conclusion using methods (3) and (4), and Wöhlisch et al. ('43) demonstrated an elegant quantitative cross

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check between various methods. Again Wöhlisch and Clamann ('32) using methods (3) and (4) showed that the tension of tendon was enthalpic. This was confirmed by Meyer and Ferri ('36), who further demonstrated that tendon could be converted into a system with entropic tension by heating strongly enough to break cross bonds (allowing contraction) taking care first to incorporate enough heat-stable ($—CH_2—$) cross bonds to prevent solution. Lundgren et al. ('48) found [method of (2)] a parallel behavior in synthetic keratin fibers, i.e., after strong heating the entropic tension became positive; these authors also made an interesting and, so far as we are aware, the first study of the effect of various substances upon the tension. Finally, Bull and Gutmann ('44) and Bull ('45) made a very careful study (method of equation 2) of the elasticity of hair, paying special attention to time effects and reversibility considerations; their conclusion was that in hair the tension is enthalpic.

shredded (as indicated by the reversibility shown below), and the thermoelastic behavior can be studied without difficulty.

It will be assumed in what follows that the structural unit of the actomyosin thread is a bundle of cross-linked parallel polypeptide chains, the filament, and that such filaments are in turn cross-linked into a network which is the thread. The qualitative nature of these cross-links will be of importance in interpreting the mechanical properties of the thread, and, apart from this, is an interesting issue both in muscle function (Morales, '48) and in protein aggregation generally. From the partial[7] solubilizing effect of .6M K it is obvious that protein-protein salt links must play an important part in maintaining the network. The issue is whether or not other bonds — specifically, hydrogen bonds — are also involved in the cross-linking. A suggestion in the affirmative is implicit in the fact that the N-H stretching frequency band of actomyosin is broad, and occurs (see below) at a frequency ($3290\ \mathrm{cm}^{-1}$) characteristic of hydrogen-bonded amides (Richards and Thompson, '47). An independent and somewhat more satisfactory confirmation was attempted in the following way. It was supposed that if the protein were cross-linked by H-bonds, then a "strong" H-bond forming substance added to the protein gel would interfere with the protein-protein H-bonding, and therefore with the maintenance of the actomyosin network. (It was found that powerful H-bonding substances do accomplish the final clearing of actomyosin-KCl solutions.) Furthermore, the degree of interference would parallel its ability to H-bond. A measure of the ability of a substance to form H-bonds was assumed to be the wave length shift, $\Delta\mu$, which the substance induces in the fundamental O-D stretching vibration of D_2O and CH_3OD (Gordy, '41a, '41b). The ability of the substance to solubilize the network was assessed by direct electron microscopic observation of protein-bonder mixtures and by observation of solubilizing action on threads.

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[7] The ratio of side chainless: non-polar: polar residues (see Lloyd and Garrod, '48 for a discussion of this ratio in relation to cross-bonding in other proteins) for actomyosin is 1: 10: 20; about half of the polar residues are acidic, and the remainder include both basic and hydroxyl side chains. Presumably K^+ weakens the electrostatic attractions between acidic and basic residues. The result of this action is not a clear solution, however.

A series of 13 substances, having fairly simple molecular structures and ranging from very poor to very good H-bonders, was selected from Gordy's lists.[8] To this selection was added 6M-urea, which on structural grounds should be an especially good H-bonder and which is known to reduce reversibly (Snellman and Erdos, '48) the viscosity of extracted actomyosin.

Experimental. The extraction and three-cycle purification of actomyosin followed in general, conventional procedure (Greenstein and Edsall, '40; Szent-Györgyi, '47), except that unusual care was taken in maintaining low temperatures (4°C. or less), purity of water, etc. A few useful refinements were also introduced: The initial alkaline brei was filtered through several layers of washed glass wool. The filtrate was precipitated in 10 volumes of water which had been previously acidified with acetic acid so as to bring the subsequent mixture to a pH between 6.5 and 6.6. After three successive precipitations, the final solution was cleared, following the suggestion of Jordan and Oster ('48), by high speed refrigerated centrifugation at 20,000 r.p.m. for one hour. In routine work protein concentrations were measured as optical densities (at 255 mμ) previously calibrated by Kjeldahl analyses.

Threads were extruded by means of a spinner patterned after that of Dubuisson ('43), and were left undisturbed to equilibrate at 4°C. for several hours before use.

For the electron micrographs the final protein-bonder mixture was 0.1% with respect to actomyosin, 0.6 M with respect to K^+, .066 M with respect to hydrogen bonding substance, and was buffered with phosphate to pH 7.2. A drop of the mixture was placed on a supporting SiO film and dried in a desiccator. Excess salt was then removed by repeated rinsing with water from a micro-pipette. All hydrogen-bonding substances used were best grade Eastman preparations except α-picoline, which was redistilled (b.p. fraction,

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[8] Gordy ('41b) found no correlation between $\Delta\mu$ and the dielectric constant of the substance; to the extent that (mol. wt.)$^{-\frac{1}{2}}$ can be taken as the dependence of diffusion coefficient on mass of small molecules (Frenkel, '46), $\Delta\mu$ is uncorrelated with diffusion coefficient; finally, there is no correlation between $\Delta\mu$ and molar solubility in water. Therefore, if with a graded H-bond series there is obtained a graded series of effects, this correlation cannot, in the first approximation, be attributed to causes other than H-bonding.

127°C.) from Eastman medium grade stock. Micrographs were taken on an RCA Model U electron microscope, employing a new type focusing device (Meryman, '49). Illustrations are of representative fields; a minimum of 5 widely spaced areas was examined in each case.

Thread solubility was examined by placing a short length of thread across two fixed pins and immersing it in pure bonder. With the exception of p-dioxane which freezes at 11°C. and was therefore left at room temperature, the effects of all the bonders were observed at 4°C. The low temperature was chosen since it was found that the solubility of threads in KCl solution decreases rapidly at room temperature, whereas at 4°C. threads are soluble for several days.

RESULTS AND DISCUSSION

The general results are summarized in figure 1 and table 1. By comparing the micrographs of mixtures with that of actomyosin control it is seen that, in general, inhibition of gelation increases with increasing H-bonding ability. The weakly bonding substances, from ethyl acetate to acetonyl acetone appear to have no appreciable effect on gelation, whereas the strongly bonding substances, from ethylene glycol to urea, show considerable inhibition of gelation. Among the intermediate substances there are some irregularities. Pyridine and α-picoline show no effect on gelation in the electron micrograph series, although they are ranked as fairly good H-bonders and dissolve threads immediately. In the thread-solubility series the general trend of increasing solubilization with increasing H-bonding ability is apparent. However, diacetyl, which is low in the bonder series, dissolves the thread instantly, whereas ethylene glycol and propylene glycol, which are relatively good bonders, fail to dissolve the threads completely.

The important paper of Lloyd and Garrod ('48) calls attention to the fact that some substances break only specific types of hydrogen bonds.[9] If the inter-filamentary bonds are quali-

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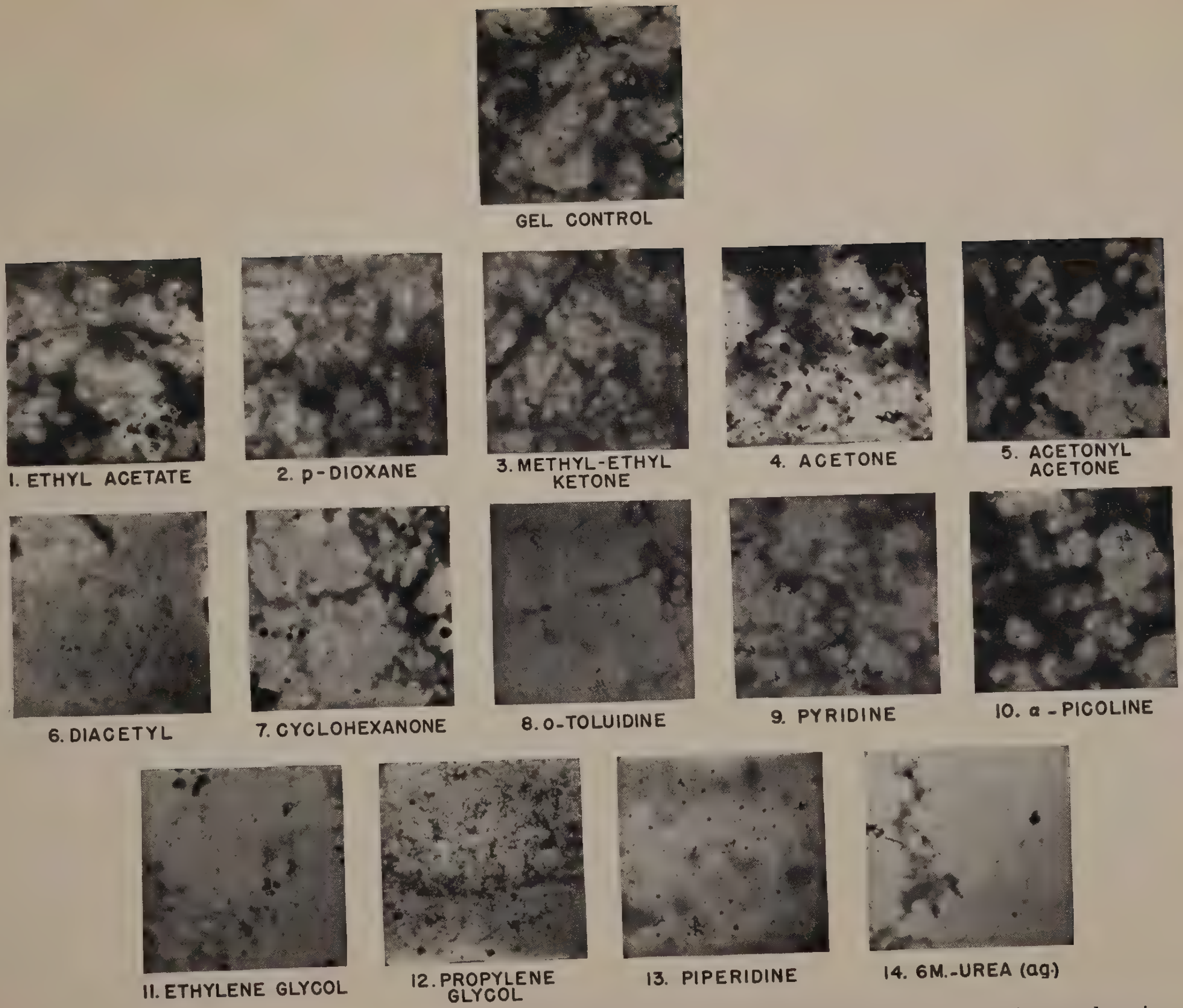
[9] For example, these authors state that formic acid is capable of breaking up (backbone) carbimino linkages, but not hydroxyl-hydroxyl linkages. In preliminary observations we have found that a solution of actomyosin in 0.6 M-KCl is not cleared by formic acid, presumably indicating that hydroxyl-hydroxyl bonds are important in maintaining the network.



GEL CONTROL

1. ETHYL ACETATE

2. p-DIOXANE

3. METHYL-ETHYL KETONE

4. ACETONE

5. ACETONYL ACETONE

6. DIACETYL

7. CYCLOHEXANONE

8. o-TOLUIDINE

9. PYRIDINE

10. α-PICOLINE

11. ETHYLENE GLYCOL

12. PROPYLENE GLYCOL

13. PIPERIDINE

14. 6M.-UREA (aq.)

Fig. 1 Electron micrographs of actomyosin in the presence of various H-bonding substances. Higher numbers indicate stronger H-bonding substances.

TABLE 1

Experiments on the interaction of various hydrogen bonding substances with actomyosin gels and threads

SUBSTANCE	$\Delta\lambda_{D_2O}$ fund. = 3.59 μ	$\Delta\lambda_{CH_3OD}$ fund. = 3.73 μ	MOLAR SOLUBILITY IN WATER	$(\text{M. WT.})^{-\frac{1}{2}}$	EFFECT ON GELS AS SEEN IN THE ELECTRON MICROSCOPE	EFFECT ON THREADS
1. Ethyl acetate	.21	.12	.965	.106	No effect	Threads become thinner but do not dissolve
2. p-Dioxane	.21	.14	∞	.106	No effect	No effect
3. Methyl ethyl ketone	.22	.11	4.87	.118	No effect	Threads become thinner but do not dissolve
4. Acetone	.22	.14	∞	.131	No effect	No effect
5. Acetonyl acetone	.22	.12	∞	.0937	No effect	Threads become slightly thinner but do not dissolve
6. Diacetyl	.24	.13	2.90	.108	Gelation reduced	Threads dissolve instantly
7. Cyclohexanone	.27	.17	.245	.101	Gelation reduced	Threads become thinner and banded
8. *o*-Toluidine	.38	.26	.14	.0966	Gelation reduced	Threads dissolve in 9 minutes
9. Pyridine	.38	.27	∞	.112	No effect	Threads dissolve instantly
10. *α*-Picoline	.40	.29	Very soluble	.104	No effect	Threads dissolve instantly
11. Ethylene glycol	.43	.31	∞	.127	Gelation reduced	Threads become very thin but do not dissolve
12. Propylene glycol	.43	.30	∞	.115	Gelation reduced	Threads become very thin and relax
13. Piperidine	.50	.37	∞	.108	Gelation abolished	Threads dissolve instantly
14. 6M —Urea (aqueous)	?	?	20.0	.129	Gelation abolished	Threads dissolve instantly
15. $\frac{6}{10}$ M —K^+Cl^- (aqueous)					Control	Threads dissolve instantly

tatively different from the intra-filamentary, then in electron micrographs a misleading result could be obtained with a reagent which breaks only the former, since the drying process would pile up filaments giving the superficial appearance of an unaffected network; this could conceivably be the basis for the few discrepancies noted above. If, however, the inter-filamentary links were simply chains shared by two filaments (as has been suggested by Ardenne and Weber, '41) then of course the hypothesis is not tenable. As noted above, pyridine and α-picoline are the only substances definitely out of line in the electron micrograph series. It is perhaps significant that these two substances have boiling points some 70°C. lower than those of substances immediately following and preceding them in the series. In the process of drying the preparations, the pyridine and α-picoline may evaporate rapidly leaving very little reagent to act on the protein. In regard to the thread solubilization series, it should be noted that o-toluidine, ethylene glycol, and propylene glycol (which show sluggish or incomplete solution of the threads) have relatively high freezing and boiling points and may therefore be sufficiently self-associated to be comparatively inert toward the protein.

In general, however, it would seem that despite these irregularities in the effects of the bonders, there is a definite trend of increasing ability to disperse the actomyosin network, and that this ability parallels the H-bonding power of the substances. The dispersion may be accomplished partially by breaking of inter-filamentary H-bonds. However, even the network of filaments visible in salt solution specimens (controls) is itself no longer distinguishable after treatment with strong H-bonding substances. This suggests that filaments are maintained intact by one or more types of H-bonds which can be broken by means of strong H-bonding substances. We can therefore visualize a filamentous actomyosin network maintained by inter-filamentary salt-linkages and by both inter- and intra-filamentary H-bonds.

In the present work the entropic tension was measured by the method of (2), i.e., by measuring $T\ (\partial\tau/\partial T)_{p,L}$, and the thermoelastic apparatus was therefore a device for measuring tensions as a function of temperature, for various extensions. It was found that information tentatively corroborating the thermodynamic results was obtainable from a study of the near-infrared spectra of unstretched and stretched threads. Certain technical details of the two measurements are described together below.

Experimental: Thermoelastic measurement. An actomyosin thread 1–2 cm long was mounted vertically between two glass hooks in a rectangular glass cell filled with .001 M phosphate buffer at pH 6.8. The upper hook hung on the balance arm of a 25 mg (vernier, .01 mg) Roller Smith torsion balance and the lower hook was firmly screwed to a brass support outside of the cell (fig. 2). The support was adjustable in three directions; in the vertical direction changes were made with a fine pitch screw so that the length of the thread could be finely adjusted. The cell was mounted inside of a heavy-walled lucite bath of about 2 li capacity; water from this bath was exchanged continuously with that in a large constant temperature bath. The apparatus was in a room maintained at 25°C. and 65% RH. Thread length and width measurements were made with a Gaertner measuring microscope arranged to sight through the bath and a flat optically ground face of the cell. Bath temperatures were measured with a mercurial thermometer marked in divisions of .1°C. The difference between bath and cell temperatures was measured by a copper-constantan thermocouple (balanced against a suitable control circuit) connected to a very sensitive galvanometer. The upper support for the thread consisted of a fine glass shaft hooked over the balance arm. The upper, hooked portion of the support was made relatively thick to prevent bending; the central portion, which passed through the surface of the solution in the cell, was made relatively thin to minimize surface tension effects. The lower submerged portion enclosed several millimeters of platinum wire to increase the moment of inertia about the balance arm and reduce swaying. The weight of this support when partially submerged was such as to allow the tensions to vary over the central range of the balance. In order to take into account the variation of surface tension with temperature, the support was weighed at various temperatures for fixed levels of submersion [10] (fig. 3).

[10] Occasionally, despite rigorous precautions, dust particles carried by the air reached the glass-air-water boundary and seriously disturbed weighing; in such instances the disturbances usually could be eliminated by shifting to a different submersion depth.

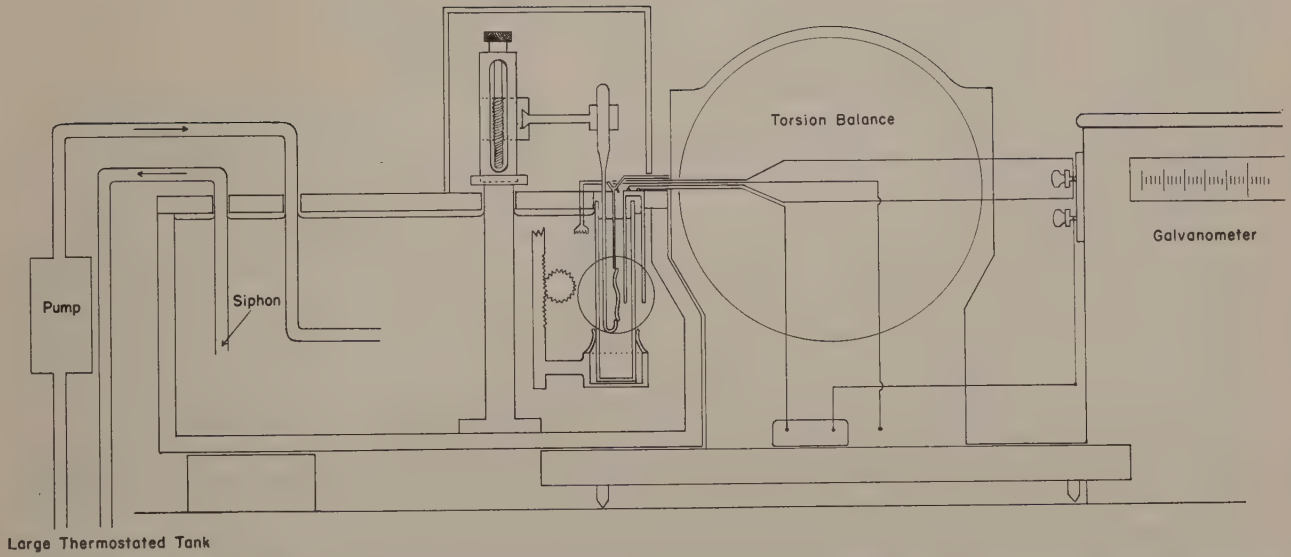


Fig. 2a Diagram of apparatus used in making length-tension-temperature measurements on actomyosin threads. Measuring microscope is focused on area indicated by smaller circle.

For mounting, a thread about 2 cm long was cut with fine glass rods and transferred by pipette to the cell filled with de-gassed water. One end of the thread was allowed to dry onto the tip of the inverted lower support, along the direction in which tension would eventually be applied, while the body of the thread remained submerged. The support was then turned right side up and clamped to the adjustable brass support. The free end of the thread was then dried onto the tip of the upper, platinum-weighted support which was suspended from the balance arm. All of the foregoing operations were carried out at about 4°C. The preparation was then aligned for optical observations and the temperature was raised to 9°C.

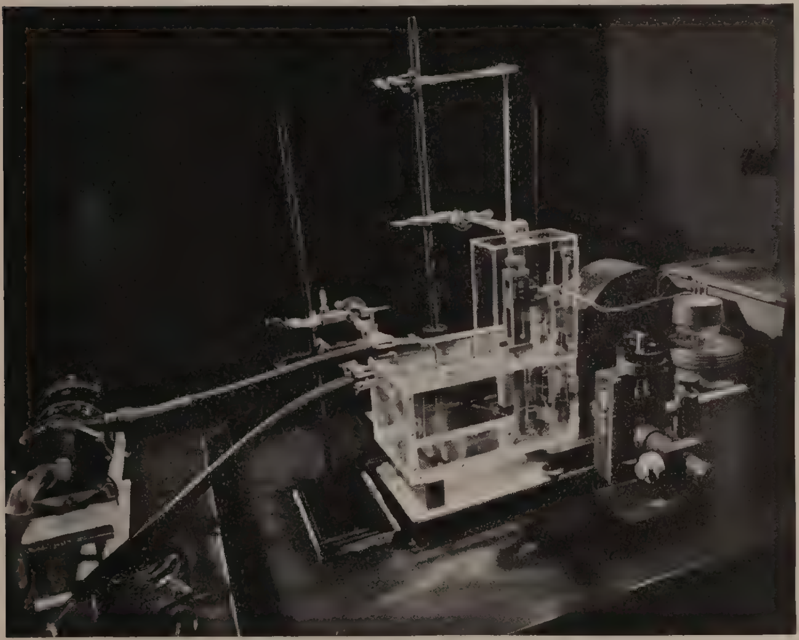


Fig. 2b Photograph of apparatus shown in figure 2a.

With the thread extended so that there was very little slack, the hook plus the unstressed thread were weighed. The lower end of the thread was then lowered by small increments and the balance reading taken after each change. When an increase in balance reading finally occurred the thread was assumed to be under slight tension. The "rest length" was then estimated to lie between the first stressed length and the immediately preceding unstressed length; this estimate was good to about 0.2% of rest length. The weight of upper support and unstressed thread were subtracted from subsequent tension readings to obtain the value of the tension exerted by the thread.

Following measurement of rest length, the thread was extended at 0.35% of rest length per minute to approximately 103% of rest length. This order of rate was adopted in order to avoid any shredding which might accompany sudden changes in length. The tension reading with time was followed until successive readings — some two hours apart — gave no further change in tension. This usually took between 10 and 20 hours (fig. 4). The relaxation was considerably faster at high temperatures, and for this reason was conducted at the high end of the 2°–9°C.[11]

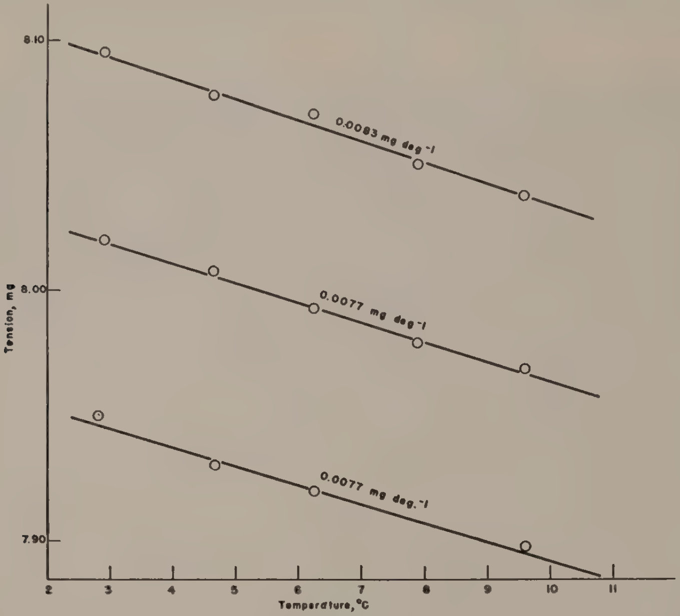


Fig. 3 Effect of temperature on air-hook-water interfacial tension. Calibration curves for upper, thread-suspending hook at three known levels of submersion.

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[11] The tension decay curves in the present work can be taken merely as assurances that equilibria were reached; it is planned to analyze them in a subsequent paper. As can be seen from the illustration (fig. 4), time effects in fibers of this sort are very marked and protracted. The reader is referred to the valuable discussions of Bull ('45) in regard to the equivalent mechanical circuit for hair keratin, and of Lundgren et al. ('49) in regard to the rate theory treatment of relaxation of synthetic keratin fibers.

After the tension leveled off the temperature was lowered rapidly some 3 or 4°. Tension measurements were then made at the new temperature until successive readings fell within experimental error (.01 mg); this in general occurred within an hour following the temperature change. After the final reading was taken at the second temperature, the temperature was again lowered 3° or 4° and the procedure repeated. Readings were thus made at 9°, 5.5°, and 2°C. After the 2°C. reading was made the temperature was raised and readings were made again at 5.5° and 9°C. as a reversibility check. Following this, the length was increased to about 105% of rest length, and the thread again relaxed over a period of several hours; when equilibrium was reached the temperature cycle was undergone. The entire procedure was then repeated 110% of rest length.

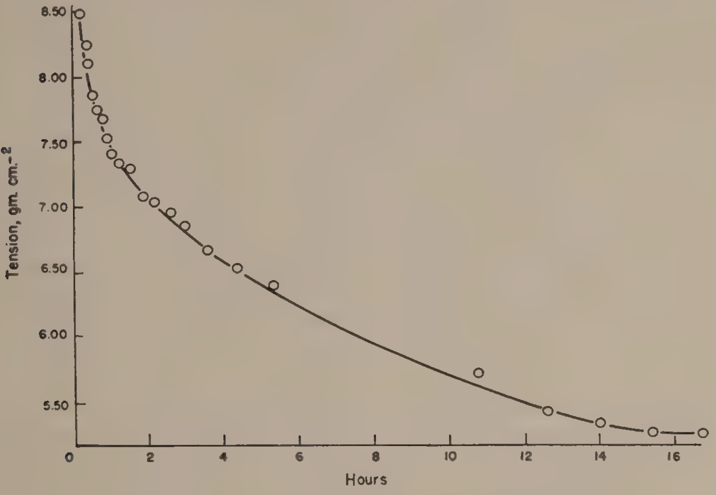

Fig. 4 Typical relaxation curve for "native" actomyosin thread, 102.9% of rest length, 9°C.

The threads described above will hereafter be referred to as "native" threads. Threads which we shall hereafter call "vulcanized" threads were made (for thermoelastic study) by attaching the ends of a "native" thread to the same supports used before, and allowing the thread to dry in air at about 150% of rest length. The thread was then immersed in phosphate buffer in the rectangular glass cell, and, after an equilibration period, tensions were measured as before. The "vulcanized" threads used for the infrared absorption studies were initially dried at about 150% of rest length, and were then reimmersed and re-stretched to about 250% of rest length; then dried again.

Experimental: Infrared absorption. For spectroscopic examination native threads were held at rest length between the pins of vernier calipers, and were then either transferred directly into a close and parallel band array on a rectangular slab of thallium bromide-iodide ("KRS-5"), or else they were first stretched to 250% of rest length and then similarly arranged. To make the scatter loss small relative to the absorption, some three layers were usually deposited. The slab was then clamped to a suitable holder; the entire system could be positioned accurately and reproducibly in the beam of the spectrophotometer. The blank was the same KRS-5 crystal. "Vulcanized" threads were taped (also in band array) over the aperture of an aluminum foil frame. The band of threads was pressed (Morales and Cecchini, '50) into a film, and the frame was then fixed over the aperture of a suitable holder. The blank was air.

Spectra in the 3 μ region were obtained at 25°C. and 35% RH with a Perkin-Elmer Model 12-B recording infrared spectrophotomer (converted to A.C. amplification) using an NaCl prism.

It is not practical to try to measure accurately the thickness of protein films usually prepared for spectroscopic examination, and it is therefore difficult to compare a "control" spectrum plotted as I_0/I with a modified spectrum plotted in the same way when the two preparations are unequal in thickness. This difficulty was overcome to a large extent by following a suggestion of Dr. K. S. Cole, viz., expressing the absorption as the lo-log (I_0/I).[12] When Beer's Law is exactly obeyed, this function is equal to a function of wave length alone plus a function of thickness and concentration. If the absorption of two preparations has exactly the same frequency dependence everywhere, then, regardless of thickness differences, the two spectra are superimposable by a translation parallel to the lo-log axis; conversely, if in some region one of the spectra has been modified relative to the other, they will fail to superimpose in that region when they are superimposed elsewhere. In practice this device is made approximate, but still highly useful, by failures of Beer's Law due either to scatter loss or to dichroism in the specimen (cf. Commoner, '49). Although mildly birefringent, native threads show little or no infrared dichroism so that it is permissible to record their spectra as just described; "vulcanized" threads, on the other hand, are intensely birefringent, and since we have not had the chance to examine their dichroism, their spectra are given in conventional fashion. (The lo-log comparison also shows a marked difference in spectra.)

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[12] The term ''lo-log'' has been introduced by Chappell ('15) to designate ''logarithm of a logarithm;'' useful tables of the function have been prepared by that author. This representation of absorption data has been employed in the past (e.g., Shurcliff and Stearns, '49), but apparently not for the purpose of overcoming this special difficulty.

RESULTS AND DISCUSSION

Results representative of the thermoelastic behavior of native threads are given in figures 5, 6, 7, and 8, and in table 2. All tension measurements are referred to unit cross-sectional area, and length measurements are given as per cent extension. The variation of rest length with temperature was less than 0.1%. The experimental value for $(\partial\tau/\partial T)_{p,L}$ at each extension was taken from the slope of the best straight line through the experimental points (fig. 6). Since there can be slight variations in protein concentrations from series to series, the resolution of the net tension into enthalpic and

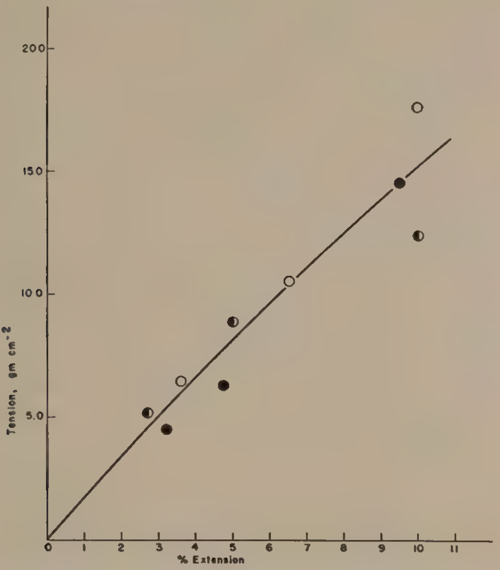

Fig. 5 Length-tension curve for ''native'' actomyosin thread at 6°C. Results for three different threads are represented by open, filled, and partially filled circles.

entropic contributions is best made by giving the enthalpic and entropic "fractions" i.e., by dividing equation (1) through by τ (fig. 7, and last two columns of table 2). Over the range of extensions studied it was found that: (1) positive tension was due entirely to the entropic contribution, (2) the

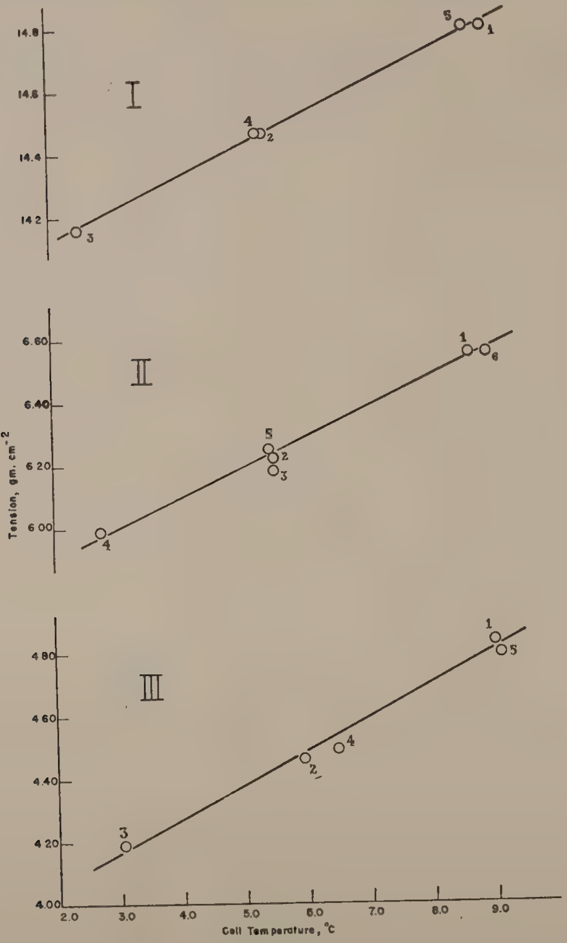

Fig. 6 Tension-temperature curves for "native" actomyosin thread at three different extensions: I, 109.8%; II, 104.9%; III, 103.1% of rest length. Small arabic numerals indicate order in which points were taken, point 1 being the first read in each case. Thread used here is represented by solid circles in figures 5 and 7.

enthalpy made a negative contribution to the tension, and (3) stretch had no effect on the near infrared (hydrogen stretch region) spectrum of ''native'' threads.

These results can be taken to mean that the tension of native threads arises, at least in part, from the kinking tendency of long chain molecules with free rotation — we specify *in part* because some length-dependent process other than un-kinking must also be contributing to the entropy; otherwise $-T(\partial S/\partial L)_{p,T}$ should increase with the length. Adopting assumption (6), we suggest further that the negative energetic contribution arises from the energy of electrostatic repulsion between charged groups of like sign along the molecular chain; this notion is in keeping with the expected preponderance of negative charges on the alkaline side of the isoelectric point

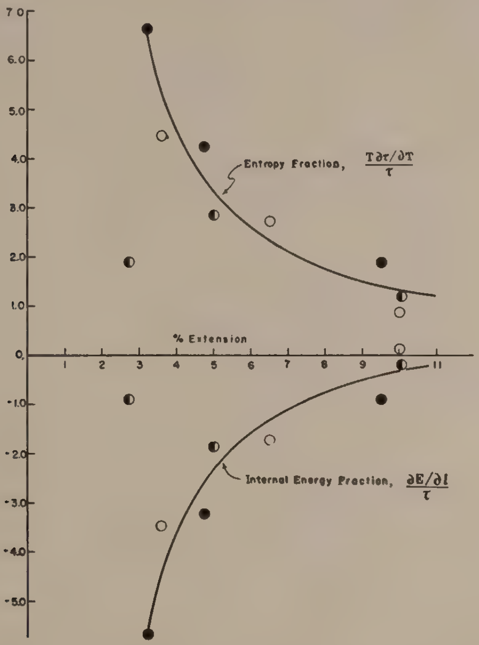

Fig. 7 Fractions of the tension in ''native'' actomyosin threads due to entropic and enthalpic (or energetic) contributions.

(pH 5.4)[13] and also with the fact that $(\partial E/\partial L)_{p,T}$ becomes less negative with stretch. Finally, the absence of a change in the infrared spectrum around 3 μ is consistent with the hypothesis that in stretching the actomyosin network bond angles or distances involving H-bonds are not altered.

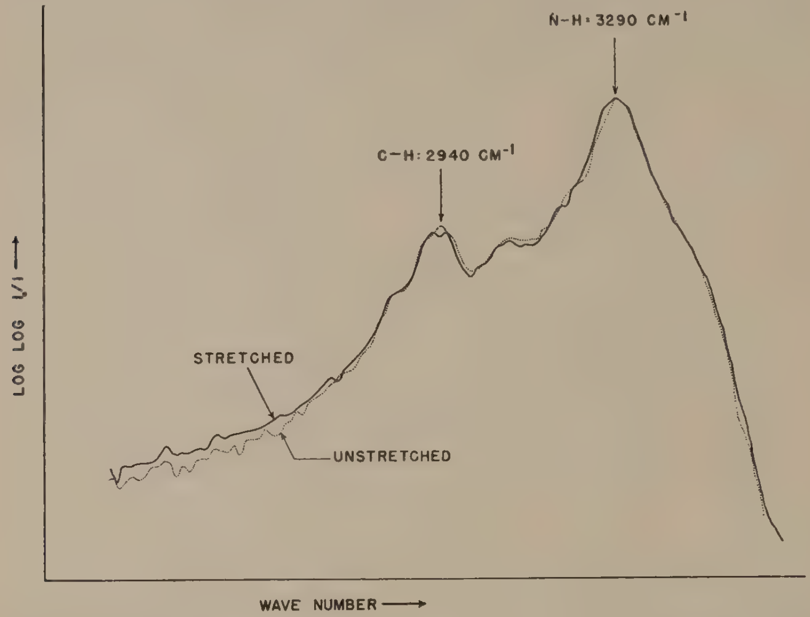

Fig. 8 Infrared spectra in the 3 μ region for "native" stretched and unstretched threads.

Results reported in the earlier part of this paper suggested that, in the course of drying, a great deal of cross-linking of actomyosin filaments was possible by H-bonding. Such a system, if dried under stretch, might conceivably show a perturbation of the near infrared spectrum. An effect of this kind was in fact found by Mr. L. E. Daasch working in our laboratory with strips of dried actomyosin film re-wet under stretch.

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[13] Graphs published by Lundgren et al. ('48) also indicate a negative contribution of the enthalpy. In a private communication Dr. Lundgren informs us that in his synthetic feather keratin fibers the tension falls on either side of the isoelectric point, which is what one would expect on the basis of our interpretation.

Daasch found that in such specimens the N-H · · · O stretching frequency at 3295 cm^{-1} (assigned by Darmon and Sutherland, '49 to *trans* H-bonds in certain amides) appeared to be reduced in height and broadened. In preliminary work, using the threads described above as ''vulcanized''—i.e., vulcanized by H-bonding upon drying—we confirmed Daasch's finding (fig. 9). As already explained a lo-log plot is probably not justified, but it will be noticed that the C-H stretching absorptions around 2940 cm^{-1} are comparatively unaffected by the stretch; i.e., the effect appears to be specifically on the N-H absorption. Because of the unavoidable crudeness of the infrared technique in studying deformation of protein films, evidence based on its results alone could not be regarded as conclusive; it did, however, suggest doing the corresponding thermodynamic experiment. The result (fig. 10) was, in contradistinction to the behavior of ''native'' threads, that in ''vulcanized'' threads the entropic contribution was negative, and the enthalpic (necessarily) positive. This second type of behavior is in agreement with Woods' work on films ('46). Our tentative interpretation is that the combination of drying and stretching allows the comparatively loose random

TABLE 2

Tension-temperature data for ''native'' actomyosin threads

PER CENT EXTENSION	τ gm cm^{-2} at 6°C.	$T\left(\frac{\partial \tau}{\partial T}\right)_{p,L}$ gm cm^{-2}	$\left(\frac{\partial H}{\partial L}\right)_{p,T}$ gm cm^{-2}	$\frac{T}{\tau}\left(\frac{\partial \tau}{\partial T}\right)_{p,L}$	$\frac{1}{\tau}\left(\frac{\partial H}{\partial L}\right)_{p,T}$
2.96	5.16	9.8	—4.6	1.89	—0.89
5.0	8.85	25.2	—16.4	2.85	—1.85
10.0	12.37	14.08	—2.4	1.20	—0.20
3.1	4.49	29.9	—25.4	6.64	—5.64
4.9	6.28	26.6	—20.3	4.24	—3.24
9.8	14.51	27.4	—12.9	1.89	—0.89
3.8	6.44	28.7	—22.3	4.46	—3.46
6.3	10.52	28.7	—18.2	2.73	—1.73
10.0	17.59	15.4	2.2	0.88	0.12

network to "vulcanize" into a semi-ordered array. Extension of this array then presumably results in a decrease of the entropy and (by straining of cross links) an increase of the internal energy.

Astbury ('48) has cited Woods' ('46) results on actomyosin films as evidence against the proposals of Wöhlisch ('40) and Bull ('46) to the effect that the contractile force in muscle may be of an entropic nature. While there is no reason to doubt Woods' results (indeed they are confirmed by our own observations on a comparable system — "vulcanized" threads), and while Astbury may eventually be proved correct, his contention now would seem to be premature, since

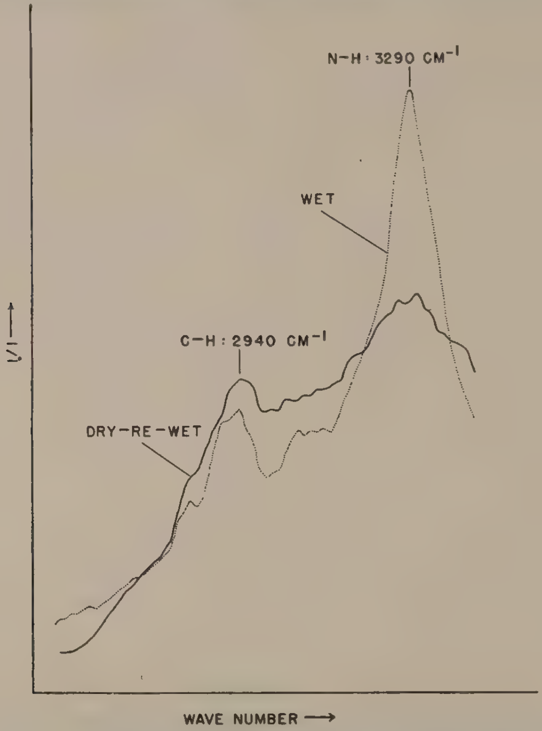

Fig. 9 Infrared spectrum in the 3 μ region for stretched, dried re-wet ("vulcanized") threads (solid line). Spectrum for undried threads (dotted line) is shown for comparison.

under certain circumstances (“native” threads) actomyosin can be made to exhibit an entropic elasticity. Existing information does not seem to justify a guess as to which of the above systems more closely resembles the intrafibrial situation; it is true that the intrafibrial concentration of actomyosin is high and that of water is low — a situation favoring “vulcanization” by H-bonds; on the other hand the concentration of K⁺ is high, thus discouraging “vulcanization” by salt linkages.

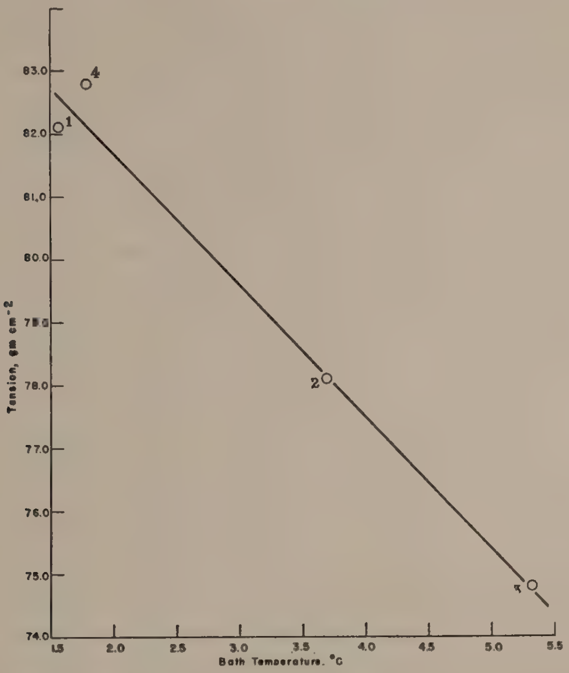

Fig. 10 Tension-temperature curve for “vulcanized” thread at 103% of rest length.

It is interesting to note that several proposals (Riseman and Kirkwood, ’48; Katchlsky, ’49; Kuhn, ’49), which appeared while this work was in progress, postulated for the “resting state” of muscle a situation identical with that deduced above for native threads, i.e., a positive entropic ten-

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sion balanced by an outwardly directed enthalpic (electrostatic?) force. If in such a system the charges are abolished the system contracts under the influence of a purely entropic tension; the model is attractive because it provides a theory for the nature of the force as well as for the "lock" which holds the fiber extended. Riseman and Kirkwood ('48) speculated that the charging process might be accomplished by phosphorylating hydroxy residues. Evidence for this process has not been found in infrared studies; an alternative phosphorylating mechanism has been suggested elsewhere (Morales and Cecchini, '50); the basic idea, however, is plausible. The suggestions of Katchalsky ('49) and Kuhn ('49) based on model experiments with polyelectrolyte gels depend on the ionizing properties of COOH groups; this mechanism likewise is *a priori* a plausible one in actomyosin.

From the recent work of Hill ('49b) it would appear that the net thermal effect in the contraction process is a release of heat. The adiabatic contraction of a system with *purely* entropic elasticity is, from equation (4), endothermic; however, in the behavior of the native thread or of the polyelectrolyte gels just discussed, the ΔH of the charge neutralization (or reduction) must be added in. The net of the two contributions may very well be negative; furthermore, the rate of heat production would be greatest at the beginning, more or less as Hill ('49a) finds. There is no question but what models and theories based on *in vitro* work must be shown to be consistent with myothermic results; on the other hand, models and theories based on myothermic work must also be plausible from the point of view of molecular structure.

SUMMARY

1. H-bonds appear to play an important role in cross-linking actomyosin.

2. The positive tension, for small extensions of "native," undried actomyosin threads, is entirely entropic; the enthalpic contribution is negative. In threads which have been dried

once under stretch (and presumably "vulcanized" by H-bonding) the positive tension is enthalpic and the entropic contribution is negative.

3. The behavior of "native" threads can be interpreted on the assumption that they have an essentially entropic tension, which is ordinarily opposed by the electrostatic repulsion of ionized groups on the chain molecules. This is precisely the assumption made in recent hypotheses about the resting state of muscle.

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THE RELATIONSHIP OF THE CELL SURFACE TO METABOLISM

V. THE ROLE OF URANIUM-COMPLEXING LOCI OF YEAST IN METABOLISM[1]

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TWELVE FIGURES

In previous papers of this series (Rothstein and Larabee, '48; Rothstein, Frenkel and Larabee, '48), it was shown that uranyl ion forms a reversible, but highly undissociated complex with specific loci on the surface of the yeast cell. The quantitative relationship between uranium uptake and the consequent inhibition of fermentation indicated that some of the surface-loci were directly involved in fermentation, while others were not.

Because uranium acts on specific, metabolically important loci on the cell-surface, any clarification of its mechanism of action may lead not only to a further understanding of the pharmacology of uranium, but also to an understanding of the relationship of the cell-surface to metabolism. It has generally been accepted in the past that the cell-membrane exerts its influence on cell function primarily through its property of selective permeability. For example, it has been suggested (Barron, Muntz and Gasvoda, '48) that uranyl ion decreases the permeability of the cell to glucose by combining with the proteins of the cell-membrane. On the other hand, an alternative hypothesis can be suggested which involves

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the active participation of the cell-surface in the metabolism of glucose. Glucose would not diffuse freely through the cell-membrane into the interior, but would be actively transported into the cell, first undergoing chemical alteration by specific enzyme reactions at the surface. Uranium would act not by reducing the permeability of the membrane, but by inhibiting certain of the surface enzyme systems. Such a hypothesis is not unreasonable. It has already been shown, for example, that specific enzymes are located on the cell-surface of yeast. These include various phosphatases (Rothstein and Meier, '48, '49) and di- and tri-saccharide-splitting enzymes (Wilkes and Palmer, '32; Myrback and Vasseur, '43; Rothstein and Meier, '50a). It is also generally accepted that in the gut and renal tubule, at least, glucose can be actively transported across cell membranes (Höber, '45). Although the specific mechanisms involved are not well established, the cell-membrane undoubtedly plays an active role. Possibly the first steps in glucose metabolism of yeast are analagous to, or similar to, the "active transport" of glucose in the gut and renal tubule, with participation in each case of the cell-membrane in specific enzyme reactions. In this regard it is interesting to note that uranium is a nephrotoxic agent which, in the whole animal, exerts its action specifically on the proximal convoluted tubule. The tubular functions which are most sensitive to uranium are those concerned with active absorption or excretion, including the resorption of glucose (Dounce et al., '49).

Although a very large volume of literature is available dealing with permeability and with "active transport," (see reviews of Brooks, '47; and Höber, '45) it does not lead to any unequivocal conclusions regarding the role of the cell-membrane in sugar metabolism. Studies of the permeability of membranes to glucose have been made, but these have been confined, for technical reasons, to cells which do not rapidly metabolize the sugars, such as algae, fungi, the higher plant cells, erythrocytes, and marine eggs. In general, these cells are relatively impermeable to glucose with one outstanding exception, the erythrocyte of humans and monkeys. Recently, however, LeFevre ('48) has found that the uptake of glucose by human erythrocytes is not a simple diffusion process, but rather, involves an active metabolic transport. In the case of cells which actively metabolize glucose, it is technically unfeasible to make a determination of the permeability of the membrane to glucose. Unfortunately, permeability relations of cells which do not metabolize glucose cannot rigorously be applied to cells which do.

Some attempts have been made to calculate whether the glucose molecule could possibly diffuse rapidly enough to account for the high rate of glucose metabolism in the yeast cell. Slater and Sand ('10), for example, calculated that the diffusion of free glucose into the yeast cell would be rapid enough to account for metabolism. However, Slater and Sand ignored the existence of the plasma membrane which might well, and probably does, impede such free diffusion. Such calculations are not subject to direct proof and one has no way of evaluating the accuracy of the calculations. Nord and Weichertz ('29) found that when yeast suspensions were stirred rapidly, the rate of fermentation was increased. They suggested that the effect was due to the increased penetration of substrate into the cell interior due to maintenance of a greater concentration of substrate across the cell-membrane. On the basis of certain assumptions, they calculated from their data the permeability of the cell-membrane to glucose. However, effects of stirring could be explained equally well by assuming that glucose is actively transported into the cell and that stirring prevents depletion of substrate in the immediate vicinity of cell-surface enzymes.

Nord and Weichertz, in order to explain the changes in rate of fermentation with increasing glucose concentrations, suggested that the substrate concentrations above 0.5 M produced a decrease in permeability by withdrawing water from the cells and their membranes. However, Hopkins and Roberts ('35) showed that the relationship between glucose or fructose concentrations and the rate of fermentation was typical of an enzyme reaction with an excellent agreement with the Michaelis and Menten ('13) equation. Gottschalk ('44) confirmed these results and concluded that the relationship between glucose concentration and the rate of metabolism represented the slowest step in metabolism, in this case the reaction between glucose and the enzyme hexokinase. However, such kinetic studies merely indicate that an enzyme step is the slowest step in the chain of reactions. They do not show whether or not the enzyme reaction is preceded by a diffusion of glucose into the interior of the cell.

Existing data concerning the role of cell-membranes in "active transport" are no more conclusive than those for permeability. There seems to be no doubt that glucose is transported across cell-membranes in the gut and in the renal tubule, but specific mechanisms are not established (Höber, '45). The same is true for phosphate, whose uptake seems to be closely associated with the metabolism (Kamen and Spiegelman, '48). It has been suggested, for example, on the basis of isotope studies that phosphate uptake in liver and muscle involves the formation of sugar phosphates at the cell-membrane (Sacks, '48) and that phosphate uptake in sea urchin eggs involves the formation of adenosine triphosphate at the cell-surface (Lindberg, '50). In a number of other cases, mechanisms for the transport of materials in or out of the cell have been postulated which involve the active participation of the cell-membrane, but unequivocal experimental verification has been difficult to obtain due to inherent technical difficulties involved in the study of such mechanisms.

In the present and following papers in this series, the initial reactions in glucose metabolism have been studied in yeast with special reference to the role of the cell-membrane. The data are admittedly not complete or unequivocal, but they provide a sufficient basis for suggesting that the first enzymic steps in the metabolism of glucose take place on the surface of the cell, and that uranium inhibits metabolism by interfering with these steps. The present paper is devoted primarily to a study of the effects of uranium on the various pathways of metabolism, using a number of substrates. The relationship of uranium-inhibition to the classic scheme of metabolism is discussed.

METHODS

Fresh Baker's yeast (Standard Brands) was used in all experiments. The yeast was washed twice in distilled water to remove extraneous material. In separating the cells from the first wash water, centrifugation was slow, sufficient only to remove about 85% of the yeast, so that light cells and cell-fragments were discarded. This procedure was found to give uniform, live cells, which were easy to centrifuge. The yeast was then suspended in distilled water, usually in a concentration of 10 mg (wet weight) per milliliter of suspension. This suspension was then starved with aeration for two hours to bring it to the constant phase of endogenous metabolism (Stier and Stannard, '37) before use in any experiment. The pH of the suspension and of all other solutions was then adjusted to pH 3.5 with HCl or NaOH.

A pH of 3.5 has been used in our laboratory for nearly all experiments with uranium for the following reasons. A yeast suspension actively metabolizing glucose in the absence of potassium maintains itself at about 3.5 even in the absence of external buffers (Rothstein and Enns, '46). In the absence of substrate, there is, at worst, a slow leakage of base with a consequent rise of a few tenths of a pH unit in several hours. The self-buffering of yeast at about pH 3.5 is of considerable advantage. At more alkaline pH's a number of complications interfere with the studies. Firstly, bicarbonate, resulting from metabolic CO_2, accumulates in the medium and forms a non-toxic complex with uranyl ion. Secondly, the organic acids (acetate, lactate and pyruvate) used as substrates in some of the experiments form non-toxic complexes with uranyl ion. Thirdly, a number of ion-species of uranium exist including UO_2^{++}, $(UO_2 \cdot OH)^+$, $UO_2\ (OH)_2^\circ$ among others. The hydroxyl complexes are relatively insoluble and are less toxic. At pH 3.5, fortunately, the above complications do not interfere. Bicarbonate does not accumulate, the complexes of uranium with the organic acids listed above are almost completely dissociated, and the only ion-species of uranium is UO_2^{++}, which is quite soluble (Dounce et al., '49).

Buffers are avoided because most of the physiological buffers effective at pH 3.5 such as phosphates and organic acids form non-toxic complexes with uranyl ion. Whenever a complexing agent is present in the medium of a yeast suspension, the uranium is partitioned between the cell-surface loci and the complexing agent in a ratio which depends on their relative concentrations and their dissociation constants (Rothstein and Meier, '50b). In consequence the expected inhibition of metabolism does not occur.

Manometric experiments were performed using a standard Warburg apparatus. Chemical procedures included: glucose, method of Hassid ('37); glycogen, method based on that of Good, Kramer and Somogyi ('33); phosphate, method of Fiske and Subbarow as modified by Meyerhof and Oesper ('47); uranium, isotope technique of Rothstein, Frenkel and Larrabee ('49).

RESULTS

Booy ('40) found that concentrations of uranium as low as 10^{-5} M markedly inhibited the fermentation of glucose by yeast as measured by CO_2 production. In a more extensive paper, Barron, Muntz and Svoboda ('48) found not only an inhibition of fermentation but an almost equal effect on oxygen consumption. These results were readily confirmed. Figure 1 shows that a uranium concentration of 1×10^{-4} M causes a reduction of the anaerobic CO_2 production to zero, and of the aerobic O_2 consumption to a low level, about equal to the rate of endogenous metabolism. In view of the inhibition of both anaerobic CO_2 production and of O_2 consumption, it is not surprising that uranium should prevent the utilization of glucose by any pathway. It was found previously (Rothstein, Frenkel and Larabee, '48) that, under anaerobic conditions, almost no glucose disappears from the medium of a yeast suspension in the presence of uranium. Under aerobic conditions the same was also found to be true.

Although uranium almost completely inhibits both aerobic and anaerobic metabolism of glucose, the actual inhibition curves are not identical. In figure 2 the inhibitions in per cent as determined by Warburg technique are plotted against uranium concentration for an experiment in which aerobic and anaerobic metabolisms were measured simultaneously on yeast from the same suspension. Before considering the data of figure 2 it should be pointed out that in the case of O_2 consumption corrections have been made for endogenous metabolism. In figure 1 it was indicated that even with high uranium concentrations, inhibition was not complete, but there remained a residual rate of oxygen consumption about equal to the rate of endogenous metabolism. This suggested that endogenous metabolism was insensitive to uranium. Such

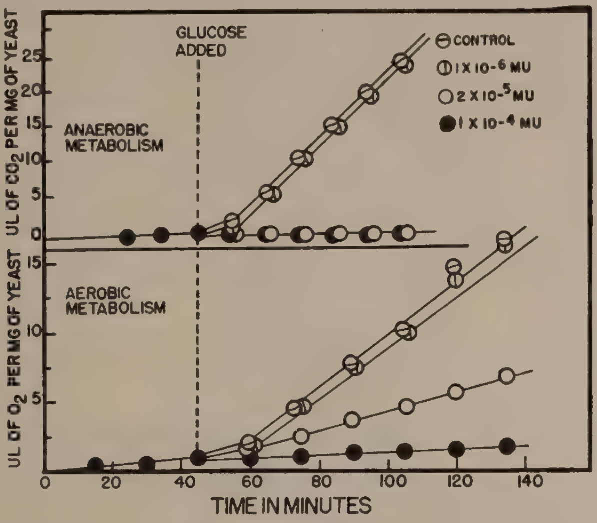

Fig. 1. The inhibition of respiration and fermentation of glucose by uranyl nitrate.

Yeast concentration was 6.5 mg wet weight per milliliter of suspension. Glucose, 0.2 M, pH 3.5 and temperature, 25°C.

proved to be the case. Figure 3 shows that concentrations of uranium as high as 1 × 10^{-2} M had no appreciable effect. In order to evaluate the effect of uranium on exogenous, aerobic metabolism of glucose it was therefore necessary to subtract the endogenous rate from each determination. The correction was necessary even when exogenous metabolism was not inhibited, for Winzler ('40) found that endogenous metabolism continues in the presence of exogenous metabolism.

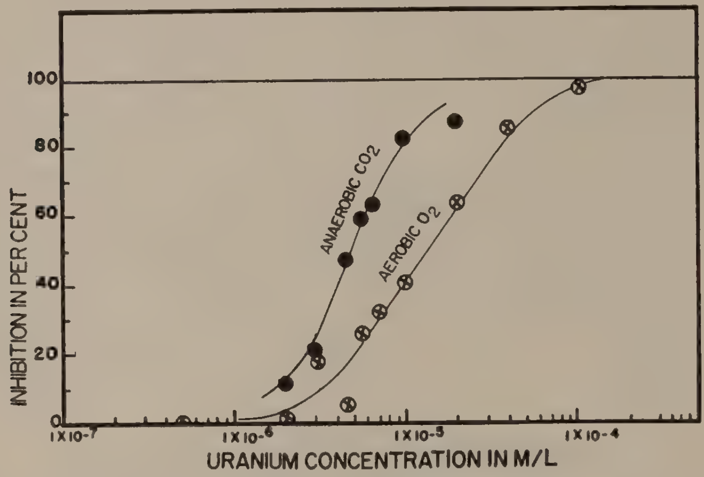

Fig. 2 A comparison of inhibition of respiration and fermentation of glucose, by uranyl nitrate.

Yeast concentration was 6.3 mg wet weight per milliliter of suspension. Glucose 0.2 M, pH 3.5 and temperature, 25°C.

The actual correction used in figure 2 was 0.7 µl of O_2 per milligrams of yeast per hour (µl/mg/hr), the average rate of endogenous respiration in 6 experiments. Although the correction amounts to only about 5% of the normal exogenous rate, in the presence of uranium it becomes rather appreciable due to inhibition of exogenous metabolism.

In the case of CO_2 production under anaerobic conditions, it was unnecessary to make any correction, because, in yeast there is no endogenous fermentation (Stier and Stannard, '36). Thus in figure 1, with a high concentration of uranium, inhibition was essentially 100%.

From figure 2 it can be seen that fermentation is considerably more sensitive to uranium than is aerobic metabolism. In order to ensure that a real difference was involved, a number of additional experiments were carried out (4 anaerobic and 6 aerobic), including three in which anaerobic and aerobic rates were determined on the same yeast suspension. The anaerobic experiments gave rather reproducible inhibition curves, whereas those done under aerobic conditions showed more variation. However, in each case aerobic metabolism was less sensitive to uranium by about the same factor. Approximately twice the concentration of uranium was required to achieve 50% inhibition of aerobic metabolism as of anaerobic metabolism.

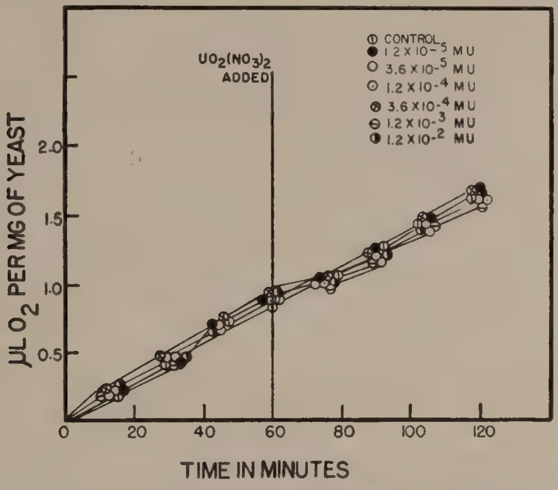

Fig. 3. The effect of uranyl nitrate on endogenous respiration.
Yeast concentration was 6.3 mg wet weight per milliliter of suspension, pH 3.5 and temperature 25°C.

The greater sensitivity of fermentation to uranium was also implied by studies of the respiratory quotient (R.Q). Normally Baker's yeast respiring glucose has an R.Q. somewhat greater than one. Even in the presence of air and with considerable agitation of the Warburg flask, some aerobic fermentation takes place. It was found that low concentrations of uranium cause a reduction in R.Q. from a normal level of 1.4 to approximately 1.0. Thus aerobic fermentation is abolished preferentially. If fermentation and respiration were equally effected by uranium no such change in R.Q. would be expected.

It has been shown previously, that under anaerobic conditions, each uranium concentration is associated with the uptake of a given amount by certain cell-surface loci according to a simple mass law equation. A given uranium uptake is associated in turn with a given inhibition of fermentation (Rothstein, Frenkel and Larabee, '48). At first, it seemed possible that the difference between the inhibition of respiration and of fermentation, shown in figure 2, might be due to differences in the uptake of uranium under the two sets of conditions. However, this did not prove to be the case. The uranium uptake as a function of uranium concentration was found to be as same aerobically as anaerobically (fig. 4). In figure 5, the actual uranium uptake is plotted against the inhibition and it can be seen that the curves are quite different for aerobic and anaerobic metabolism.

TABLE 1

The effect of uranium on dinitrophenol stimulated, endogenous fermentation

	CO_2 PRODUCED IN 70 MINUTES		CO_2 PRODUCED IN 70 MINUTES
	µl		µl
Control	0	DNP 1×10^{-3} M + U	105
Control	—3	DNP 1×10^{-3} M + U	107
		DNP 5×10^{-4} M + U	101
DNP 1×10^{-3} M	96	DNP 1×10^{-4} M + U	88
DNP 1×10^{-3} M	92		
DNP 5×10^{-4} M	98		
DNP 1×10^{-4} M	101		

The yeast concentration was 40 mg per flask, at pH 3.5 and 30°C. Uranyl nitrate concentration was 1×10^{-4} M.

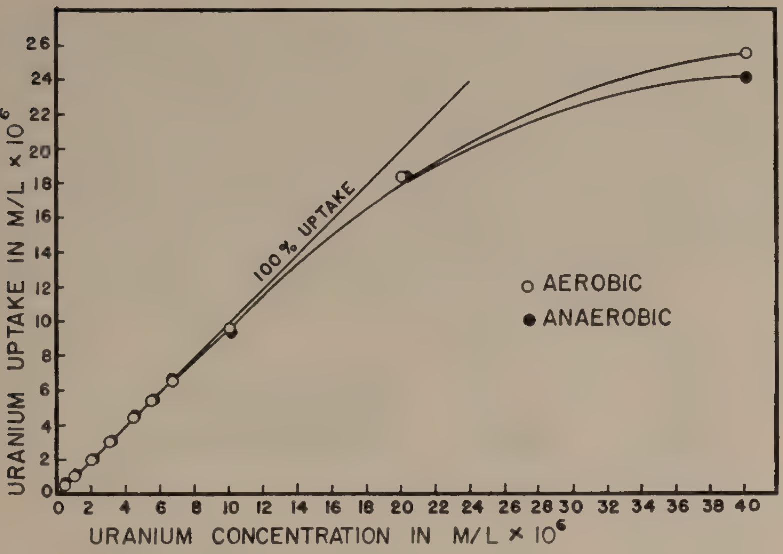

Fig. 4. Uranium-uptake by the cells as a function of uranium concentration. Yeast concentration was 6.3 mg wet weight per milliliter of suspension, pH 3.5 and temperature 25°C.

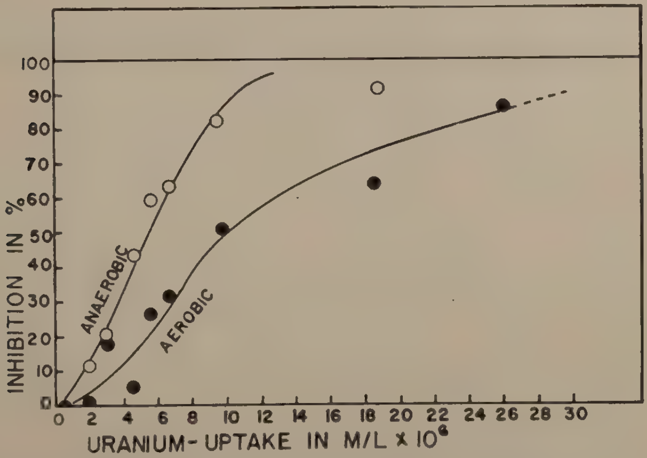

Fig. 5 The relationship between uranium uptake and inhibition of respiration and fermentation of glucose.

In the experiments of figure 1 the uranium was added to the yeast suspension 10 minutes before the addition of glucose. The inhibition was immediately apparent. Figure 6 shows that the same was true if the uranium was added at the same time as the glucose. If, however, the uranium was added 10 minutes after the glucose, there was a delay of some 30 minutes before any inhibition was observed, and of some 60 minutes before inhibition was maximal. The lag period which occurs when glucose is added prior to uranium is probably associated with the utilization of intermediates of glucose metabolism which accumulated in the cell prior to the addition of uranium. Uranium apparently does not interfere with the utilization of such intermediates but does prevent their further formation by inhibiting the initial steps in glucose metabolism. The time-delay suggests that an early step in metabolism is the one inhibited by uranium.

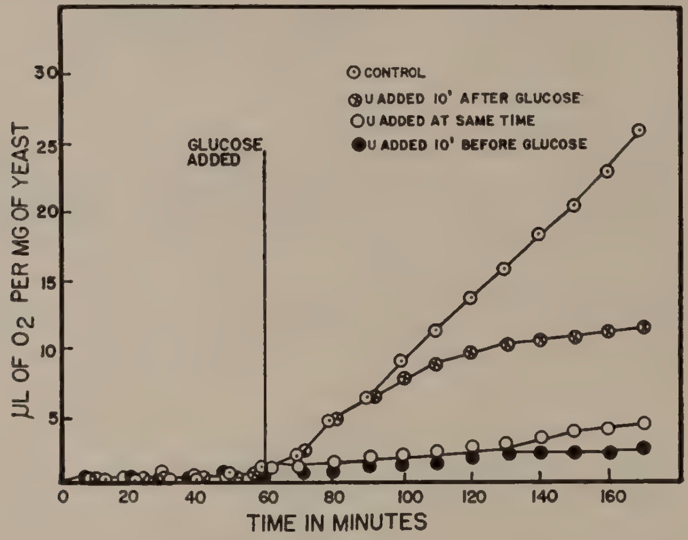

Fig. 6 Time delay in inhibition when glucose is added before the uranyl nitrate.

The yeast concentration of 6.5 mg wet weight per milliliter of suspension. Glucose 0.2 M, pH 3.5, temperature 25°C., and uranyl nitrate 1×10^{-4} M.

The uranium effect is not limited only to the inhibition of glucose metabolism, but applies to that of other hexoses. For example, fructose fermentation is inhibited by approximately the same concentrations of uranium as is glucose metabolism (fig. 7). Galactose is not normally fermented by yeast, but if yeast cells are allowed to oxidize glucose in the presence of galactose for several hours, they acquire the ability to ferment galactose at a high rate. The adaptation of yeast to galactose involves the formation of ''adaptive enzymes'' required to ferment this substrate (Spiegelman, Lindegren and Hedgecock, '44). Uranium in low concentrations completely inhibits the fermentation of galactose by adapted yeast (fig. 8). Thus uranium seems to be a particularly good inhibitor of the metabolism of the hexoses.

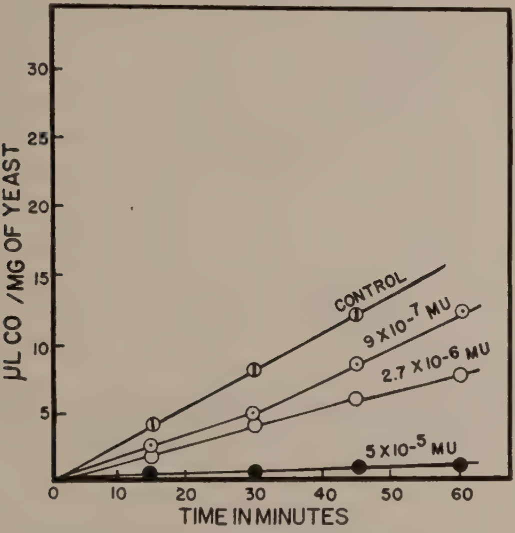

Fig. 7 The inhibition of fermentation of fructose by uranyl nitrate. The yeast concentration was 5 mg wet weight per milliliter of suspension. Fructose 0.1 M, pH 3.5, and temperature 25°C.

The effect of uranium on the metabolism of a number of other substrates was tested. As pointed out in the section on Methods, it is necessary to know whether or not a given substrate forms a complex with the uranyl ion at the pH used in the experiments. If a complex is formed, then the uranium will be partitioned between the cell loci and the substrate in accordance with the mass law, the amount of uranium available for inhibition of metabolism being thereby reduced. Because substrates are present in high concentrations they may combine with nearly all the uranium, with the result that no inhibition is observed even though uranium is capable of combining with the enzyme systems involved. For example, in figure 9 it is shown that citrate, which forms a relatively stable complex with uranyl ion, completely reverses the inhibition of glucose metabolism. It would be difficult therefore to determine whether or not uranium can combine cell-groups involved in citrate metabolism.

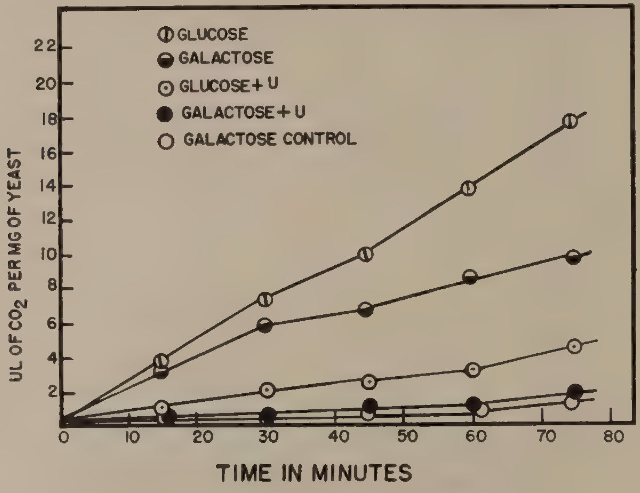

Fig. 8 The inhibition of fermentation of galactose by adapted cells. The yeast concentration was 5 mg wet weight per milliliter of suspension. Sugars 0.1 M, pH 3.5, temperature 25°C., uranyl nitrate 5×10^{-6} M. The cells were adapted to galactose by aerating for two hours in a mixture of glucose plus galactose. They were then washed twice, starved for 30 minutes, and the galactose added.

At pH 3.5, the pH used in these experiments, a number of substrates do *not* complex strongly with uranium. These include ethyl alcohol, pyruvate, acetate and lactate. This was tested by studying the effect of these substances on the ferrocyanide-uranium color. At high pH's however, the organic acids can form complexes (Dounce et al., '49) which would reduce the amount of uranium available for inhibition.

In figure 10, the inhibition of respiration by uranium is given for each of the substrates listed above. For comparative purposes the curve for respiration of glucose is included. The respiration of alcohol and pyruvate is not inhibited by 1×10^{-4} M uranium, which is sufficient to completely inhibit the respiration of glucose. Even at 1×10^{-3} M uranium, respiration of alcohol is only inhibited about 10%, and pyruvate not at all. On the other hand, the respiration of acetate and lactate is inhibited about 20% at 1×10^{-4} M uranium, and 35 to 40% at 1×10^{-3} M. It must be pointed out that the same 35–40% inhibition of glucose respiration is achieved by about 5×10^{-6} M uranium, only one-two-hundredth as high a concentration. Thus the uranium-complexing loci concerned

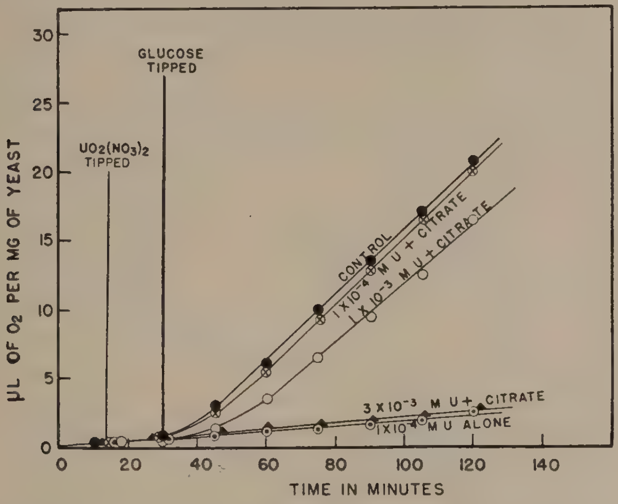

Fig. 9 The reversal of uranium-inhibition by citrate.

The yeast concentration was 6 mg wet weight per milliliter of suspension. Glucose 0.1 M, pH 3.5, temperature 25°C., and sodium citrate, 0.01 M.

with glucose metabolism have no importance in the metabolism of alcohol and pyruvate, and probably little importance in the metabolism of acetate and lactate.

The uranium inhibition was further characterized by studies of glycogen synthesis and degradation. During starvation (endogenous metabolism) glycogen is consumed at a low rate through pathways which are different from those involved in glucose metabolism. The endogenous metabolism only takes place in the presence of O_2 (Stier and Stannard, '36). Endogenous metabolism measured either in terms of O_2 consumption (fig. 3) or glycogen disappearance (fig. 11) is quite insensitive to uranium in concentrations sufficient to completely inhibit glucose metabolism. Thus, the endogenous pathways are not affected by uranium.

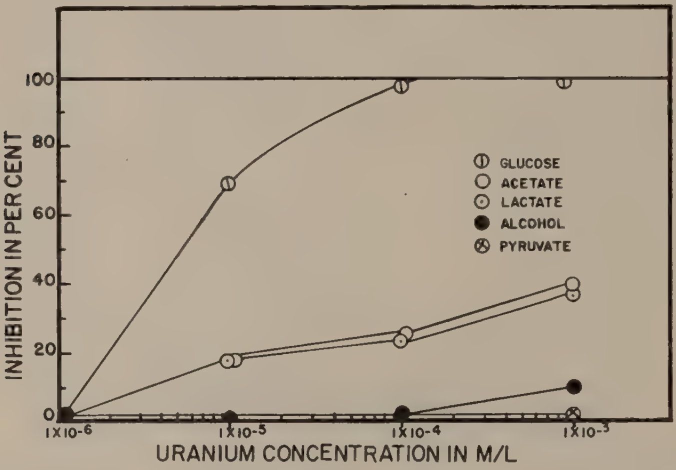

Fig. 10 The effect of uranyl nitrate on the respiration of various substrates. The yeast concentration was 5 mg wet weight per milliliter of suspension. Substrate concentrations 0.01 M, pH 3.5, and temperature 25°C.

The synthesis of glycogen from various substrates presumably involves the formation of glucose-1-phosphate which is converted to glycogen by the enzyme phosphorylase (Green and Cori, '43). With uranium present, glucose is not utilized and this substrate cannot, therefore, be used to test the effect of uranium on glycogen synthesis. However, as shown previously, uranium has little effect on metabolism of alcohol and this substrate will normally give considerable glycogen formation (Brücke, '33). Figure 11 shows that 10^{-3} M uranium has little effect on glycogen synthesis from alcohol, although this concentration of uranium completely inhibits glucose metabolism. If it is assumed that glycogen formation from alcohol involves in essence a reversal of the steps of fermentation then we may conclude that uranium has no effect on the steps of this system.

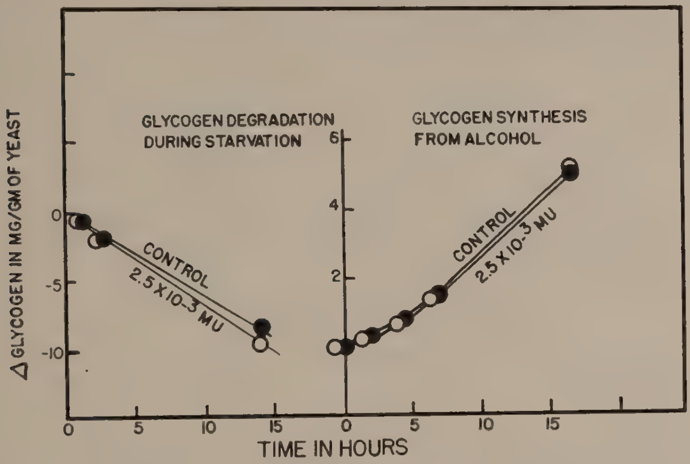

Fig. 11 The effect of uranyl nitrate on glycogen synthesis from alcohol The yeast concentration was 40 mg wet weight per milliliter of suspension. Alcohol 2% by volume, pH 3.5, and temperature 25°C.
and on glycogen degradation during starvation.

Further evidence that uranium does not effect the fermentation pathways is found in the following experiments. It has been shown (Reiner and Spiegelman, '47) that dinitrophenol allows a rapid mobilization of newly formed glycogen in yeast. Rothstein and Berke ('50) found that this mobilization was accomplished by the coupling of glycogen degradation into fermentation pathways. Normally glycogen is utilized only aerobically, at a slow rate. In the presence of adequate concentrations of dinitrophenol, glycogen is utilized anaerobically with the production of CO_2 and alcohol. Thus dinitrophenol can activate the fermenation pathways even though no external substrate is present. Table 1 indicates that uranium in concentrations sufficient to inhibit glucose metabolism has little effect on the dinitrophenol enhanced fermentation of internal stores.

DISCUSSION

If the generally accepted scheme of carbohydrate metabolism is examined in detail with reference to the uranium effects, it is apparent that uranium can act only on steps which introduce sugars into the general metabolic system. All the other parts of the system are active in the presence of sufficient uranium to inhibit the utilization of hexoses. To illustrate this point, an abbreviated, diagrammatic scheme of metabolism is given in figure 12. The Krebs cycle, cytochrome system, and associated systems (hydrogen transport and phosphate transfer) are utilized in the aerobic metabolism of alcohol, pyruvate, acetate and lactate, which are relatively

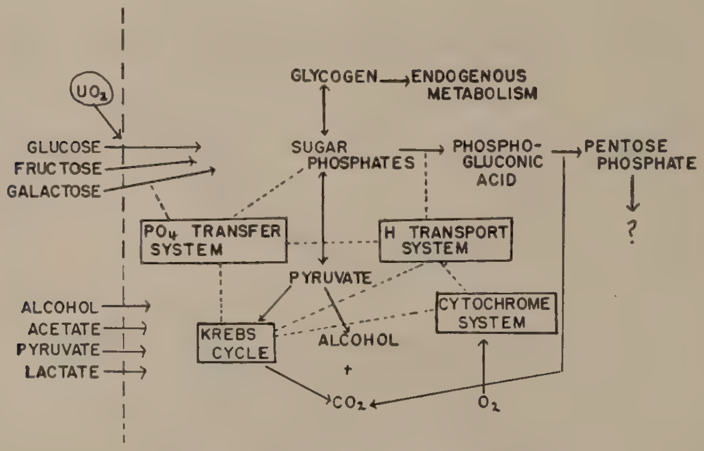

Fig. 12 Scheme of metabolism.

insensitive to concentrations of uranium which will inhibit glucose metabolism. These portions of the metabolic cycle must therefore be unaffected by uranium. The fermentation pathways from alcohol to glycogen by way of the phosphorylated sugars are presumably involved in glycogen synthesis from alcohol, and also in endogenous fermentation induced by dinitrophenol (Rothstein and Berke, '50) both processes relatively insensitive to uranium. The same is true for endogenous metabolism of glycogen, which proceeds through a different pathway than does exogenous metabolism (Stier and Stannard, '37). By elimination, the only steps in the scheme on which uranium might act are those unique to hexose metabolism, presumably those involved in the introduction of these compounds into the general metabolic pathways.

In the living cell, the events in metabolism are more complicated than indicated in the scheme (fig. 12). A very important consideration involves the relationship of cell structure to metabolism and particularly the role of the cell membrane. It has already been shown (Rothstein and Larrabee, '48) that uranium acts at the surface of the cell, and this fact is consistent with the conclusion that uranium acts on one of the earliest steps in metabolism. The first interactions between a cell and a substrate in the surrounding medium must take place at the surface of the cell. Because uranyl ion does not penetrate into the interior of the cell, this ion cannot act on processes which take place inside the cell even though it might be capable of combining with some of the enzyme systems involved. Thus studies of isolated enzyme systems do not necessarily yield information which is useful in evaluating the effect of uranium on intact cells. The uranium effect is associated with the integrity of the cell membrane and for this reason the available experimental techniques useful in studying the action of uranium are limited to those which can be applied to intact cells. In the present paper metabolic studies are presented. In following papers in this series (Hurwitz and Rothstein, '50; and Rothstein and Meier, '50b) the kinetics of uranium inhibition and the chemical nature of the cell-surface loci will be discussed. On the basis of these and earlier studies it is possible to present a general hypothesis concerning the role of the cell-surface in carbohydrate metabolism.

As pointed out briefly in the introduction, the role of the cell membrane in sugar metabolism may be a passive one, with the substrate diffusing through the membrane into the interior of the cell in response to its concentration gradient. Barron et al. ('48) suggests that this is the case and that uranium inhibits glucose metabolism by combining with proteins of the cell-membrane, thereby decreasing the cell's permeability to glucose. An alternative hypothesis involves the "active transport" of glucose into the yeast cell such as is known to take place in the gut and in the renal tubule. In the case of the yeast cell, however, it is possible to postulate a specific mechanism. It is suggested that certain enzymes which catalyse the initial reactions in the metabolism of glucose are located on the surface of the cell. The substrate would pass into the interior of the cell only after chemical alteration at the cell-surface. It is suggested that the cell-surface reactions involve phosphorylation, and that uranium prevents these reactions by chelating with necessary polyphosphate groups.

It was also pointed out in the introduction that the available literature suggests that cell-membranes are impermeable to sugars. However, the evidence is far from unequivocal, and such a generalization may be unwarranted. On the other hand, it has been definitely shown that specific enzymes are located on the cell-surface; therefore, the general concept of enzyme reactions occurring at the cell-membrane is acceptable.

When the data on uranium inhibition are examined in some detail, they seem to be readily consistent with the hypothesis that enzyme reactions are involved, but are not always consistent with the permeability hypothesis. In a previous paper (Rothstein, Frenkel and Larrabee, '48) it was found that uranium forms a specific 1 to 1 complex with certain loci on the surface of the cell. A specific number of uranium-complexing loci are associated with fermentation, and these particular loci show a first order relationship to metabolism. In other words, a proportionality exists between the percentage of the loci which are complexed with uranium and the percentage inhibition. For example, when 30% of the loci are combined, 30% inhibition results, etc. Such a proportionality implies that any particular locus combined with uranium is unable to participate in metabolism at all, whereas any locus which is not combined is able to participate in a completely normal fashion. Thus the uranium-inhibition involves a fixed number specific loci on the cell-surface, each of which responds in an all or none fashion. Such behavior would be expected if uranium were inhibiting an enzyme reaction. In the case of permeability, however, too little is known about the actual factors that would be involved in the diffusion of glucose through a cell-membrane to make any positive statement concerning the above data.

A second point worthy of discussion concerns the difference between the inhibition of aerobic and of anaerobic metabolism. Not only is aerobic metabolism less sensitive to uranium than is anaerobic metabolism, but the actual relationship of uranium-binding to inhibition is different. Thus, more uranium-binding loci are associated with aerobic metabolism than with anaerobic metabolism. At a uranium concentration which will give essentially complete inhibition of fermentation, some respiration still proceeds. In other words, when all the loci utilized for fermentation are blocked by uranium, other loci still uncomplexed by uranium are functioning in respiratory pathways. The latter loci presumably form a somewhat less stable complex with uranium so that the uranium preferentially combines with the fermentation-loci. At any rate, there seem to be different species of uranium-complexing loci involved in fermentation and in respiration. This conclusion is strongly supported by studies reported in the next paper of this series (Hurwitz and Rothstein, '50), in which it is shown that the kinetics of inhibition of fermentation by uranium are quite different from the kinetics of inhibition of respiration.

The differences between respiration and fermentation are quite compatible with the hypothesis that the initial steps at the cell-surface are enzymic in nature. It would only be necessary to postulate that some divergence occurs between aerobic and anaerobic pathways at this early stage in metabolism, so that different enzyme systems are utilized having different affinities for uranium. However, in the case of permeability, aerobic-anaerobic differences are harder to explain. The process inhibited by uranium should be the same in each case, namely the penetration of glucose through the cell-membrane by diffusion. There should presumably be no differences in the number or character of the loci.

The strongest evidence in favor of the enzyme-hypothesis is given in the following papers of the series. They can only be discussed briefly here. In the first of the papers (Hurwitz and Rothstein, '50) it is shown that not only are the kinetics of uranium inhibition different under aerobic and anaerobic conditions, but that in each case, the kinetics are typical of those expected in enzyme reactions, not of diffusion-limited processes. In addition, it is shown that the temperature characteristics of the uranium-inhibited reactions are quite high, of the order of 22,000 calories per mol, a value consistent with an enzyme reaction rather than a diffusion process.

The other paper (Rothstein and Meier, '50b) is concerned with the chemical nature of uranium-complexing loci. It is concluded that the surface loci involved in sugar metabolism are some kind of polyphosphate. Uranium forms a very stable complex with such groups by a ring closure, possibly replacing calcium and magnesium which might be normally present. It is much more probable that polyphosphate groups participate in metabolic reactions such as phosphorylations, rather than serving as a permeability site for the diffusion of glucose into the cell.

SUMMARY

1. Uranium, as uranyl ion, in low concentrations, acts specificially on the metabolism of the hexoses, including glucose, fructose, and galactose in adapted cells, preventing utilization of these substrates by anaerobic or aerobic pathways. Quantitatively, in terms of uranium uptake, the effect of uranium on aerobic and anaerobic metabolism is different. Aerobic metabolism is less sensitive to uranium.

2. There is a time factor involved in the inhibition of glucose metabolism. If the uranium is added earlier than, or at the same time as, the substrate, the effect is instantaneous. If the uranium is added 10 minutes after the substrate, there is a considerable delay in the appearance of any inhibition.

3. Uranium in concentrations that effectively block glucose metabolism have little effect on the respiration of alcohol, acetate, pyruvate and lactate or an endogenous respiration. At considerably higher concentrations, uranium partially inhibits the respiration of acetate and lactate.

4. Uranium has no effect on glycogen degradation during starvation, nor glycogen synthesis from alcohol.

5. Uranium has no effect on endogenous alcoholic fermentation induced by dinitrophenol.

6. The qualitative effects of uranium are discussed in terms of the known cycles of metabolism and with special reference to the fact that uranium acts on the surface of the cell. A hypothesis is presented concerning the active role of the cell-membrane in metabolism and the specific action of uranium thereon. It is suggested that the phosphorylation of sugars actually takes place on the cell-membrane.

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DIFFERENCES AMONG SPECIES IN THE RESPONSE OF FIREFLY EXTRACTS TO ADENOSINE TRIPHOSPHATE [1]

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INTRODUCTION

Early investigations on light production by various luminous organisms established that a terminal oxidation of a substrate, luciferin (LH_2), in the presence of a specific enzyme (luciferase) and oxygen was the basic light-emitting reaction (Harvey, '40). Although this reaction has not been established for all species of luminous organisms, it is probable that it represents a type reaction which is basic for all examples of bioluminescence. In addition to the terminal oxidative step, it has recently been demonstrated that continued light production in cell-free extracts of fireflies depends upon an adequate supply of high energy phosphate groups (McElroy, '47). Adenosine triphosphate (ATP) is the only known labile phosphate compound which can maintain luminescence in these cell-free extracts. The results indicate that the terminal oxidative step does not supply sufficient energy for light emission and that the energy for this process is obtained from preliminary transphosphorlating dark reactions. In addition to ATP and heat labile catalysts, it has been established that in firefly lantern extracts a heat stabile, blue-green fluorescent compound (firefly luciferin) and Mg^{++} ion are essential for light emission (McElroy and Strehler, '49; Strehler and McElroy, '50).

Studies thus far indicate that when cell-free extracts are made from firefly lanterns the light disappearance depends

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upon the exhaustion of labile phosphate groups. However, we have had recently the opportunity to perform additional experiments on a number of Jamaican and American species of fireflies and have found an interesting biochemical difference. The results reported in this paper clearly demonstrate that in some species the factor which first limits light production in aqueous extracts is not alone labile phosphate groups. Light production may also be limited by the luciferin and luciferase concentration.

MATERIALS AND METHODS

The American species of fireflies were collected in and around the Baltimore area in the summer of 1949. The organisms were dried in vacuum over $CaCl_2$ and stored in the cold at — 18°C. until used. The Jamaican fireflies were collected near Kingston, during the month of January 1950. Most of the experiments reported in the present paper were performed on the fresh material immediately after catching the fireflies; otherwise the fireflies were preserved in a $CaCl_2$ canister until they were dry. The firefly luciferin was prepared and purified by chromatographic methods (Strehler and McElroy, '49), and the ATP was the sodium salt preparation obtained from Rohm and Haas, Philadelphia.

EXPERIMENTAL RESULTS AND DISCUSSION

The observations recorded in table 1 were made by removing the luminous lantern from the fireflies and grinding in a mortar with water until the light disappeared. For most species of fireflies this dark extract will emit additional light when ATP is added. For certain species of Jamaican fireflies, however, this was not the case. When ATP is added to extracts of *Photinus melanotis* or *Diphotus montanus* no light is obtained. In an effort to stimulate light production in these extracts a purified sample of firefly luciferin prepared from *Photinus pyralis* was added. In the absence of ATP no light was obtained; however, in the presence of ATP there was an excellent luminescence. The results indicate that in these two species of firefly both ATP and luciferin are limiting factors. Since both *Photinus melanotis* and *Diphotus montanus* have restricted light organs, the latter characterized by two small light spots on the ventral surface of the 8th sternite, it was felt that additional experiments on luminous organisms with small light organs would be informative. The light emitted from the small restricted organs of the glowworm closely resemble the light of *Diphotus*, and it is remarkable that, biochemically, extracts of some Jamaican and American glowworms require both luciferin and ATP for light production. Whether the restriction of light organs in luminous forms is due to the limited synthetic ability of these organisms to produce luci-

TABLE 1

The production of light by firefly lantern extracts in the presence of adenosine triphosphate and Phontinus pyralis luciferin

ORGANISM	ATP	LH_2	ATP + LH_2	FLUORESCENCE OF EXTRACT	
				Acid	Base
A. Jamaican fireflies					
1. Photinus lobatus lobatus	+	—	+	blue	blue-green
2. Photinus pallens	+	—	+	blue	blue-green
3. Photinus lobatus morbosus	+	—	+	blue	blue-green
4. Photinus commissus	+	—	+	blue	blue-green
5. Photinus melanotis	—	—	+	. .	. .
6. Diphotus montanus	—	—	+	weak blue	weak blue-green
7. Photuris jamaicensis	—	—	—	blue	blue-green
8. Glowworm (unidentified)	—	—	+	. .	. .
9. Pyrophorus (sp.) from Puerto Rico	+	—	+	. .	. .
B. American fireflies					
1. Photinus pyralis	+	—	+	blue	blue-green
2. Photinus scintillans	+	—	+	blue	blue-green
3. Photuris pennsylvanica	+	—	+	light blue	green
4. Two unidentified species	+	—	+	. .	. .
5. Glowworm (Photinus pyralis)	—	—	+	light blue	light blue-green

+ = light, — = no light, LH_2 = luciferin, ATP = adenosine triphosphate.

ferin is not clear, but certainly the results support such a hypothesis.

An additional species of firefly which fails to respond to ATP is *Photuris jamaicensis.* Furthermore, a light response cannot be obtained when both ATP and luciferin are added together. Since this species of firefly contains large luminous organs, it was suspected that adequate luciferin was present. In an effort to determine the nature of the limiting factor in the *P. jamaicensis* extracts, several cross reactions were tried. The results of these experiments are recorded in table 2. Of interest is the fact that when extracts of *P. jamaicensis* are mixed with *Diphotus montanus* a bright light is obtained even in the absence of ATP. Since *D. montanus* requires both ATP and LH_2 for light production, the results indicate that the extracts of *P. jamaicensis* contain adequate amounts of both of these substances. This is supported also by the fact that *D. montanus* extracts fail to emit light when mixed with *P. pallens* in the absence of ATP. On the other hand, if *P. jamaicensis* extracts are mixed with extracts of *P. pallens* or *P. pyralis* in the absence of ATP an excellent light is obtained. Since hot water extracts of *P. pallens* or other fireflies fail to stimulate light production in *P. jamaicensis*, it seems justifiable to conclude that an essential enzyme is destroyed when the light organs of the latter species are extracted. This is not an unlikely possibility, since it has

TABLE 2

Cross reactions of extracts from the lanterns of fireflies in the presence and absence of adenosine triphosphate

		DIPHOTUS MONTANUS	PHOTINUS JAMAICENSIS	PHOTINUS PALLENS	PHOTINUS PYRALIS
Diphotus montanus	+ATP	—	+	+	0
	—ATP	—	+	—	0
Photinus jamaicensis	+ATP	+	—	+	+
	—ATP	+	—	+	+

0 = not tested, + = light, — = no light.

already been shown that the luminous extracts of other fireflies are extremely labile to temperature and pH conditions, a fact which may explain some of the earlier observations on the difficulty of extracting the luminescent system from other organisms.

Previous studies have demonstrated that most fireflies contain a large number of blue fluorescent compounds in the light organs. One of these compounds (firefly luciferin) is essential for light production and is distinguished from the others by the fact that a green fluorescence is emitted in alkaline solutions rather than the usual blue. Observations on the fluorescence of extracts of the Jamaican fireflies support the conclusions reached above concerning the lack of luciferin in *Photinus melanotis* and *Diphotus montanus*. When first made, extracts of *Diphotus montanus* contain very little fluorescent material; however, within 3 to 4 minutes after the extraction a faint blue fluorescence appears which changes to a blue-green fluorescence when alkali is added. This blue-green fluorescent compound does not support the luminescent reaction and may, therefore, represent an inactive luciferin. Recently it has been possible to isolate from *P. pyralis* a green fluorescent compound which is not active but is very similar in physical and chemical properties to the active luciferin. It seems likely, therefore, that in the *Diphotus* extracts the luciferin is rapidly converted into an inactve form.

In addition to the 12 species of lampyrid fireflies, one elaterid firefly (Pyrophorus sp. from Puerto Rico) was tested for its ability to respond to ATP. This tropical firefly possesses a pair of circular light organs on the dorsal surface of the thorax and a single organ on the first abdominal segment. In appearance the thoracic organs emit a greenish light, while the abdominal organs emit a more orange light. When extracts of these two different organs were made it was found that ATP was active in restoring luminescence. This ability to respond to ATP was lost within 30 minutes after extracting and could not be restored with *P. pyralis* luciferin. Activity could be restored by adding extracts from the non-luminous portions of the body. The results indicate that in the crude extracts the enzyme system is rapidly destroyed.

The difficulty of demonstrating a luciferin-luciferase reaction in some luminous forms may be explained, at least in part, by the results of the present investigation. In luminous forms such as *Diphotus montanus* and *Photuris jamaicensis* it has been demonstrated that a destruction of certain necessary components of the luminous system is brought about by the extraction procedure. Alternate methods of preparing such extracts may give more favorable results for those cases in which the luciferin-luciferase reaction has been reported as negative.

SUMMARY

A study of 8 species of Jamaican and 5 species of North American lampyrids has indicated that, in addition to water and oxygen, luciferin, luciferase, adenosinetriphosphate (ATP) and Mn or Mg salts are necessary for continued luminescence in extracts of the light organs. Depending on the species of firefly, ATP, luciferin or luciferase may be the first of the extracted substances to disappear. In most Lampyrids and the Elaterid, Pyrophorus, ATP disappears first in an extract of lanterns and adding ATP to the extract will restore the light. In Photinus melanotis, Diphotus montanus and some larval forms (glow worms), the luciferin disappears first. Adding ATP will not restore the light but ATP + Photinus pyralis luciferin is effective. In Photuris jamaicensis, ATP + luciferin will not restore the light but addition of luciferase from another fire-fly is necessary.

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QUALITATIVE OBSERVATIONS ON VISIBLE CHANGES IN SINGLE FROG, SQUID AND OTHER AXONES SUBJECTED TO ELECTRICAL POLARIZATION

IMPLICATIONS FOR EXCITATION AND CONDUCTION

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ONE FIGURE

It has been reported that electrical polarization of whole, multifibered crayfish nerve results in shrinkage with increased opacity and increased light scattering in the region of the anode, accompanied by swelling and increased transparency in the region of the cathode, and that these changes are reversible, either upon standing or upon reversal of electrode polarity (Tobias and Solomon, '50a). The rather extensive older literature pertinent to this phenomenon and stemming particularly from work of Munk (1868), Bethe ('03) and Höber ('45) is quoted in the above reference and need not again be cited here. A phenomenon thought to be fundamentally similar has also been described for Elodea cells (Tobias and Solomon, '50b) and for many other cells (references given in Elodea paper). Observations have now been extended to other nerves, the effects of ions are being investigated, and the additional observation has been made that nerves polarized on Pt electrodes become more than normally rigid in the region of the anode and more than normally soft and sticky in the region of the cathode. There is also evidence that possibly related, visible changes may occur in excited cardiac ganglion cells (Fedorow, '35) and may be propagated along stimulated crab (Carcinus maenas) nerve (Hill and Keynes, '49).

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Because of possible, as yet hypothetical, implications of the observations for the general phenomena of excitation and conduction (Tobias, '51) there are several questions which must be answered in the beginning. One of these is whether the phenomenon, in whole or in part, can be demonstrated in individual axones isolated prior to polarization or whether, though it involves the single axone it does so only in the whole nerve. This communication describes certain electrically induced, polar unique changes in single axones polarized after isolation.

METHODS

Little discussion of methods is necessary. In the case of the squid (Loligo pealii), the giant axone of the stellar nerve has been isolated in sea water by excising the nerve, tying it at each end with silk and dissecting away all other fibers. Such an isolated axone, still conducting apparently normal impulses, can then easily be transferred to a pair of electrodes.

Other single axones (frog, crayfish, lobster, Carcinus maenas, spider crab) are somewhat more difficult to isolate. For polarizing on polarizable electrodes (Pt wires) one end of the whole nerve is tied with silk to one of the electrode pair. The other end of the nerve is then teased and frayed with fine forceps and needles, and finally the frayed end is draped over the second electrode. Usually, by simple chance, several individual and well separated axones are seen to be laying over and contacting the second electrode. Such dissection is much easier if carried out in Ringer solution or sea water than in mineral oil. In oil, which was tried most often with frog nerve, the individual axones strongly tend to adhere to each other and to rejoin after partial separation, suggesting that the outermost surface of the axone is better wetted by water than by oil. Similarly teased axones can be bridged across non-polarizable electrodes. Once the axone is on the electrodes the water solution can be replaced by mineral oil to minimize short circuiting.

Non-polarizable electrodes have been of two types. One has been a silver-silver chloride electrode made by chloridizing silver wire in KCl. Others have been made of cotton thread wicks soaked in an appropriate electrolyte solution and connected by agar bridges (5–6% agar in Ringer or sea water) (approx. 30 cm long) to saturated calomel half cells.

Whichever electrodes are used, whether in mineral oil or salt solution, one can, with a microscope, watch the axones directly as current traverses them. Under no circumstances, for obvious reasons, is the current through a given axone known. However, upper limits can be estimated roughly.

RESULTS

Using polarizable electrodes (Pt wire)

In the case of single frog axones the changes seen on polarization with Pt wire electrodes are reminiscent of those previously described for whole crayfish nerve (Tobias and Solomon, '50a) except that they appear to have a faster onset, seconds instead of a minute or so. When the electrode is made cathodal the axone contacting it swells and increases in transparency to the point where it may become difficult to see in bright transmitted light. The changes start where the axone touches the electrode and spread along its long axis, primarily into the interpolar region toward the anode but also somewhat into the extrapolar region. If, on the other hand, the electrode be made anodal the axone becomes more dense. It shrinks and its surface shows an irregular and tortuous wrinkling. Highlights and dark regions appear as the surface irregularities develop and the whole looks much like axones soaked in high calcium solutions as studied by Ettisch and Jochims ('27). As in the case of whole crayfish nerve, these polarization induced changes are reversible either on stopping or reversing current flow. If the current be reversed the changes remaining from the previous polarization disappear and are replaced by their opposites nearest the electrodes first. The whole reversible phenomenon may be elicited repeatedly with the same axone.

At a polarizable anode the dimensional changes of the frog axone are more complex than at the cathode. With closure of the circuit the fiber narrows transversely, much as it swells transversely at the cathode, but in addition to this transverse shrinkage there is a simultaneous longitudinal shrinkage toward the point of contact with the anode (fig. 1). It is occasionally difficult to be sure that points along the axis of the fiber do or do not approximate each other more closely during the time of current flow. Usually it is clear that they do come closer together toward the anode, but the picture is sometimes obscured by the changing surface contours. The fact of such shortening or longitudinal shrinkage toward the anode is supported, however, by an additional observation. Thus, sometimes the single fiber draped over the electrode trails off into the medium beyond it (fig. 1). Such a trailing tail clearly shortens along its long axis toward the anode. Though some part of it may lie closer to the electrode than the rest, as at

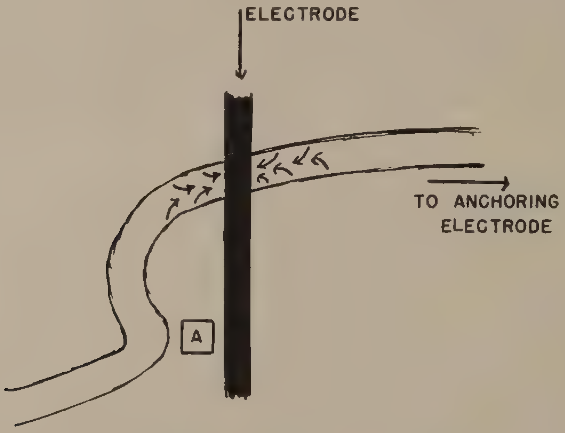

Fig. 1 Single axone trailing over electrode (purely diagrammatic). Arrows show direction of movement when electrode is made anodal.

point A in figure 1, the fiber does not visibly move sidewards to contact the electrode at A. Thus, even if there is an electrokinetic tendency for the whole fiber to move anodally, due to surface charge, some other force is sufficient to overcome this and produce longitudinal shortening toward the anode.

It is of some interest that when one watches a tail of axone trailing over an anode, as in figure 1, it often seems to shorten in a spiral fashion. After the movement has stopped the fiber may be seen to have contracted along its long axis toward the anode and to have curled up into a spiral. This may have to do only with the geometry or spatial arrangement of the axone on the electrode. On the other hand, it may imply some sort of submicroscopic spiral structure in the protoplasm (Seifriz, '47).

The frog axone anodally can undergo these dimensional changes quickly, and it is, therefore, able to execute a rapid shortening and subsequent lengthening in the region of the electrode as a result of a sharp finger tap on a circuit key. If the key is held down the fiber remains shortened. When the key is released it reelongates. This phenomenon is, in a sense, fatiguable. After a certain number of repetitions with a given fiber a rest period is required or the quick movement will no longer be elicitable. After a large number of repetitions or if the key be held down too long the phenomenon usually fails completely. No comparable rapid movement, either shortening or elongation, has been seen at the cathode.

It is not possible with such a preparation to know how much current traverses the axone, but approximation from the voltage used and from published resistance data suggests that the phenomenon occurs with as little as 0.05 microamperes. In earlier eperiments a colleague, Mr. Solomon, and the author once or twice thought they saw perielectrode movement of whole nerve on tetanization (crayfish) and Flaig ('47) has reported a similar observation with the squid giant axone.

If one now leaves the frog axone and watches certain invertebrate axones (squid, spider crab, Carcinus maenas, lobster, crayfish) similarly polarized on polarizable electrodes the consequences are somewhat different. Anodally there is reversible transverse shrinkage, opacity increase and increased rigidity (the latter observed in squid) while cathodally there is transverse swelling, transparency increase and increased stickiness (the latter observed in squid), but there is no surface wrinkling or longitudinal shrinkage, slow or rapid, near the anode as in the case of the frog axone. The meaning of this difference will be considered later. The increased rigidity at the anode causes the axone, when it is lifted, to come cleanly off the electrode as a rather stiff rodlet, while at the cathode it peels away limply and is visibly adherent or sticky. In this context it should be recalled that Pt wire electrodes in a sodium salt buffered solution such as used here cause a lowering of pH at the anode and a rise of pH at the cathode.

Using non-polarizable electrodes

Since the most numerous observations have been made using the squid giant axone on wick electrodes, the following applies to this system unless otherwise specified.

To begin with, larger voltages, times and currents are necessary to produce visible effects with wick electrodes (8–45 v.; 10–80 microamperes; 3–12 min.) than with polarizable platinum electrodes (1–3 v.; 0.05–3 microamperes; seconds). One can make little of such numbers, however, because (a) there is at present no way of knowing how much of the current goes along the surface water film or through the axone, and (b) these numbers refer to the onset of clearly visible changes whose sub-visible initial fraction may start instantaneously with minimal voltage and current.

If the axone be polarized on sea water soaked wicks, the whole submerged in mineral oil, the following changes are seen: Cathodally the axone swells and becomes more than normally transparent and turgid, anodally it shrinks and becomes opaque. The anodal transparency decrease is first seen nearest the electrode, but whether the opaque material causing it first forms at the axonal surface or inside is not known. When it is visible, however, it is seen to be inside and can be moved about, like a floating cloud in the axoplasm, by slight mechanical pressure. In many cases it seems to come loose and when this happens it moves toward the cathode. When it does so migrate it leaves an excessively clear area behind it at the anode. If the axone be now transected on the anodal side of such a migrating cloud and if the gap be bridged by a sea water soaked thread then such cathodalward migration continues. Also, under the anode one frequently sees a plasmolysis like change. Just as after fixation with alcohol, the axone or ''protoplast'' is seen to shrink down within the sheath. These changes are reversible.

If now the axone be polarized with a small pool of isotonic $CaCl_2$ or $MgCl_2$ bathing it and the cathode at their region of contact and a small pool of isotonic KCl bathing it at the anodal contact then the same sort of changes in transparency and size are seen but usually in reverse order. Now the cathode usually produces shrinkage and opaque axoplasmic cloud while around the anode there is slight swelling and increased transparency. If the axone be lifted off the electrodes and reversed in position on them the changes are reversed. This is of particular interest in view of the fact that it has not been possible to produce any change in transparency of the giant axone, either visibly or as measured photometrically, by simple immersion in isotonic $CaCl_2$ or KCl.

Turning now to the phenomenon of peri-electrode movement as was described for the frog axone in the region of the polarizable anode, it may be said that on non-polarizable electrodes, whether wicks or Ag-AgCl, neither the frog nor any of the invertebrate axones examined showed any anodal surface wrinkling, increased rigidity or longitudinal shrinkage. Nor has there been any apparent increase in stickiness around the cathode as seen with Pt electrodes. On the contrary, the axone appears to become somewhat more turgid than normal near the cathode probably due to water movement toward this region. On attempting to pick it up with forceps it tends to roll out from between the closing tips.

DISCUSSION

The phenomena of surface wrinkling and quick movement of the frog axone in the region of an anode are only seen with polarizable electrodes (Pt) and not with non-polarizable electrodes (Ag-AgCl or wicks), and are seen with neither type of electrode when invertebrate fibers (Cambarus spp., Loligo pealii, Homarus americanus, Carcinus maenas, Libinia emarg-ginata) are examined. Therefore, though the effect is of intrinsic interest (possibly depends on a structural difference between the two types of fibers as well as on some electrochemical effect of the polarizable electrodes, perhaps a pH change) it is probably not of great interest in a consideration of normal excitation and conduction. Its observation, however, highlights the fact that one knows very well but often ignores, to wit that observations made with one of these types of electrode may not justifiably be carried freely over in thinking about polarization (or stimulation) mechanisms in general.

The slower dimensional changes, anodal shrinkage and cathodal swelling transversely, are, on the other hand, seen in all fibers examined regardless of electrode type used, and are of interest and potentially of importance. One element in the mechanism of such diameter changes is almost surely electroendosmotic flow of water toward the cathode. Changes in diameter of 30–50% have been seen, and it is difficult to see how such a large diameter change could occur without water movement. Also, in other experiments (Tobias and Rudenberg, '50) such movement of water toward the cathode has been measured directly using lobster nerve.

On the basis of experiments with other colloidal systems (Bartell, '23; Höber, '45) it seems justifiable to conclude that water movement toward the cathode means that the surfaces over which it slips carry a net negative charge. This is what one would expect if the fixed cellular surfaces (inside and outside) are coated with some amphoteric material at a pH above its isoelectric point. On these same grounds one would expect that if an excess of some divalent ion such as Ca or Mg could gain access to these surfaces then the charge might well be reversed and water should flow away from a cathode in such a region. Accordingly it has been seen that when the region of contact with the cathode is bathed in isotonic $CaCl_2$ or $MgCl_2$ the cathode then can produce shrinkage as would be expected on these grounds.

Recalling now the sequence of electrical events during the passage of an impulse (Hodgkin and Katz, '49), if one makes the assumption that the propagated, longitudinally oriented e.m.f.'s which travel along the axone can do on an ultramicro level what is made obvious and visible by polarization, then one would expect a complex to-and-fro movement of water along the long axis of the nerve, in the region of the propagated disturbance at any instant. Thus, there should be, if this reasoning is correct, propagated volume changes accompanying the impulse. A purely theoretical approach to this question has been attempted by others (Fry and Fry, '51).

It is also of interest that the anodal region shrinks even though there is water available in the contiguous wick electrode. Apparently, under these conditions, water moves along the axone more readily than it can move across its surface.

The mechanism of the transparency and opacity changes is not clear. Certainly dehydration of the anodal region and excessive hydration of the cathodal one might be expected to increase anodal opacity with increased light scattering and to increase cathodal transparency with decreased light scattering. However, the notion has been entertained for some time (Tobias and Solomon, '50a, b) that the changes seen are probably due, in part, to a partial segregation and separation of certain cations by the applied e.m.f. Such a thesis postulates that the more mobile K ion moves or is oriented toward the cathode more rapidly than is the slower Ca ion. The net gain in Ca ion around the anode might then change solubility or degree of dispersion of certain macromolecules there and produce clouding, an opposite effect due to K ion excess occurring at the cathode. It has been seen, however, that, under the conditions used, simply bathing the giant axone in either isotonic KCl or $CaCl_2$ did not change transparency. Also, extruded squid axoplasm retains a sausage like shape in isotonic KCl but is dispersed by $CaCl_2$ concentrations up to at least 76 mM (Hodgkin and Katz, '49a), and the microinjection of $CaCl_2$ in concentrations up to isosmotic (Chambers and Kao, '51) has produced no visible clouding in transmitted light. These facts then do not support the notion of anodal agglomeration by way of Ca accumulation and cathodal dispersion by way of K accumulation. On the other hand, the presently reported experiments show that both Ca and Mg ions can reverse the usual effects of cathodal polarization, and in their presence, in sufficient concentration, the cathode produces clouding and shrinkage: the K ion, in turn, can reverse the usual anodal picture and prevent anodal clouding and shrinkage. This ionic reversal of visible polarization effects has also been found with whole crayfish nerve and is implicit in the results of many of the older workers (Tobias and Solomon, '50; Mackuth, '26; Schwartz, '11).

Such seeming discrepancies might be explained in several ways; that somehow the current allows the divalent ions to penetrate and exert an effect, that although the K ion may be assumed to penetrate without a current it does not alone increase transparency because it is already present in excess, that the effects of the divalent ions are exerted while passing through the surface and therefore when injected into the axoplasm they produce no apparent change, that the effects seen with whole crayfish nerve are due to actions of the ions on intercellular material, etc. It might be more profitable, on the other hand, to think about the phenomenon in terms of current plus certain ions having different effects than either one alone. At the moment the matter must be left in midair.

The whole argument, however, raises an important possibility. If, in one way or another, polarizing electrodes produce a change in degree of dispersion or particle size of certain protoplasmic colloids then they may also produce localized changes in osmotic pressure. Again, if the longitudinally oriented e.m.f. propagated with the impulse can do, on a smaller scale, what the e.m.f. applied by electrodes can do, then propagated with the impulse there should be a change in degree of dispersion of such colloids and a resultant propagated change in osmotic pressure. A visible change which might well reflect such a dispersive change has been reported to occur in frog auricle ganglion cells on vagal stimulation (Fedorow, '35) and a propagated change in transparency has been reported to occur in stimulated crab nerve (Hill and Keynes, '49). (See addendum added.)

Obviously, it is implicit in these suggestions that the changes seen at the cathode, and interpreted to imply increased colloidal dispersion and hydration there, may be the visible expression of structural degradation with increased permeability and decreased transmembrane resistance known to occur under the exciting electrode. Under the inhibiting or blocking electrode, the anode, on the other hand, opposite changes take place. Once the impulse becomes self-propagating and is conducted, the longitudinally oriented e.m.f. which is a part of it takes over the water moving, ion separating, osmotic pressure changing and colloid dispersing or agglomerating properties previously resident in the stimulating electrodes.

A final matter is the fact that the anodal cloud definitely gets into the axoplasm and then moves cathodalwards. Whether it moves cathodally by active electrophoresis due to its own charge (which would tend to become positive if an excess of Ca or some other divalent ion as Mg accumulated at the anode as postulated) or is carried there passively by electroendosmotic water flow is not now known. Whichever may be the case, if such material is formed at the "anodal end" of a travelling impulse it may tend to be carried toward the advancing "cathodal end." Similarly, other substances may move in such a travelling voltage gradient, either by direct electrokinesis or along with electroendosmotically moved water (Hearon, '50). At the moment too little is known of the duration and orientation of various electrical fields which may accompany the impulse. If there is eventually found to be a net negativity or positivity in one direction then such observations as those reported here might be said to foreshadow an explanation of the movement of material along the long axis of nerve in the direction of propagation and of certain concentration gradients which have been found in nerve (Kraus et al., '24; Roeder, '30; Gerard, '32; Gerard and Tupikova, '39; Weiss and Hiscoe, '48; Samuels et al., '49).

SUMMARY

1. Freshly dissected frog axones are surface hydrophilic and oleophobic.

2. Frog axones polarized on polarizable electrodes show anodal shrinkage (transverse and longitudinal), surface wrinkling and increased opacity, whereas cathodally there is swelling and increased transparency. These changes are reversible and can be induced repeatedly. The anodal shrinkage and subsequent relaxation may occur rapidly as a result of momentary current flow. This rapid shortening and elongation fatigues and finally fails on repetition. On non-polarizable electrodes no such quick longitudinal movements or surface wrinkling are seen. The slower transverse dimensional changes and transparency changes do, however, occur on non-polarizing electrodes also.

3. Invertebrate axones show no surface wrinkling or quick longitudinal movement around the anode whether polarized with polarizable or non-polarizable electrodes. The squid axone on polarizable electrodes (as is also true of whole crayfish, lobster and frog nerves) shows increased rigidity and lack of adherence to the electrode anodally, whereas cathodally

it becomes limp and quite sticky. This is not seen with non-polarizable electrodes.

4. The squid axone, on non-polarizable or polarizable electrodes, shows reversible transverse swelling at the cathode with increased transparency there. At the anode there is reversible narrowing and opacity increase. The opacity increase appears to be due to an opaque material which is seen as a cloud in the axoplasm which can be moved about by mechanical pressure and which migrates toward the cathode when current flows. Whether the latter migration is directly electrophoretic, accompanies electroendosmotic water flow or results from both is not yet clear.

5. Higher voltages and longer times are required to produce visible changes with non-polarizable than with polarizable electrodes.

6. In the squid axone a "plasmolysis" like phenomenon with shrinkage of the protoplasm within the sheath is often seen under the anode.

7. Using the squid axone it has been found that in the presence of a local excess of $CaCl_2$ or $MgCl_2$ the cathode produces narrowing and increased opacity rather than the usual swelling and increased transparency, and in the presence of a local excess of KCl the anode is prevented from causing the usual narrowing and increased opacity.

8. The findings suggest that surfaces (continuous or particulate) inside the squid axone are negatively charged.

9. It is suggested that the swelling and increased transparency seen under a polarizing cathode are the visible expression of structural degradation with increased permeability and lowered transmembrane resistance assumed to occur under the exciting electrode. Contrariwise the anodal narrowing and increased opacity may be the visible expression of protoplasmic structural changes responsible for blocking under such an electrode.

10. If the various interpretations are correct, and if the propagated, longitudinally oriented e.m.f. which accompanies or is part of the travelling impulse can produce on an ultra-

micro scale the sort of changes that the e.m.f. applied via polarizing electrodes produces on a visible scale then the nerve impulse may well have as component facets (a) a propagated volume change, (b) a propagated osmotic pressure change, (c) a propagated change in particle size of certain protoplasmic colloids, (d) a propagated change in transparency and light scattering, and (e) a movement of various substances along the long axis of the axone.

ADDENDUM

While this paper was in press, D. K. Hill (J. Physiol., *111:* 283–327) further described a propagated opacity change in a crustacean nerve trunk and also reported that repeated stimulation causes an initial, short-lived shrinkage followed by a later, longer lasting diameter increase in the single axone of Sepia. The swelling is thought to be due to an increase in the osmotic pressure of the interior of the fiber. How these findings correlate with the contents of this paper requires further analysis.

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SOME STUDIES ON THE INFRARED ABSORPTION OF THE CONTRACTILE SYSTEM OF SKELETAL MUSCLE *

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NINETEEN FIGURES

INTRODUCTION

Many of the reactions, known and postulated, attending the fundamental contractile process of skeletal muscle may be expected to have counterparts in infrared absorption changes. Among these are dephosphorylations of the nucleotides, phosphorylation of the contractile proteins, cysteine-cystine equilibria, alterations in hydrogen bonding of the proteins, etc. Since at its worst the infrared method is a unique tool in the qualitative analysis for molecular groupings, and at its best is an important source of mechanical information about molecules, the attractiveness of applying the method to muscle reactions is obvious. The difficulties in this application are, however, equally obvious. For example, in such an application it is necessary, although illogical, to study complicated structures before their sub-units have been satisfactorily analyzed. In addition, it is necessary to study dry systems which, in regard to relevant structures, may or may not represent the living systems. The present paper reports preliminary studies on the reaction between various nucleotides and actomyosin as well as on muscle in different states of contraction. For this work it was necessary to characterize spectrally the "pure" participants; these spectra are likewise reported, for they have intrinsic value in the elucidation of protein and organic phosphate structure. The difficulties referred to above are painfully evident in the results; nevertheless, we feel that certain phases of this study show considerable promise and advantage over alternative approaches.

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* The opinions or assertions contained herein are the private ones of the writers and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large.

EXPERIMENTAL METHODS

Instrumental. Spectra reported in this paper were obtained with a Perkin-Elmer Model 12B automatically recording infrared spectrophotometer, converted to AC amplification (beam-chopper system). An NaCl prism was employed in the region, 800–1700 cm^{-1}; an LiF prism in the region, 2900–3500 cm^{-1}. All spectra were obtained at 25°C., and a relative humidity of 35%. Taking various sources of error into account, we estimate that the frequencies cited are good to $\pm$ 5 cm^{-1} at about 1000 cm^{-1} and $\pm$ 13 cm^{-1} at about 3300 cm^{-1}. Evaporated films were deposited on windows of crystal thallium bromide-iodide, "KRS-5," (see Barnes and Gore, '49), obtained through the courtesy of the Army Engineer Research and Development Board, Ft. Belvoir. To position KRS-5 windows and other specimens in an accurate and reproducible manner, it was necessary to construct various cell-holders which could be slipped in and out of the ways of the spectrometer housing. The polarizer was a pile of 5 AgCl sheets (coated with AgS) held at 63.5° with respect to the incident beam and rotatable through 360° (Newman and Halford, '48).

For reasons described elsewhere (Botts and Morales, '50), spectra are reported as the lo-log (incident intensity/transmitted intensity) vs. frequency in wave numbers, following an original suggestion from Dr. K. S. Cole. In the absence of scatter, variation of film thickness or concentration only shifts the lo-log function parallel to the vertical axis, so that curves of the same substance are always superimposable by mere vertical translation. This becomes only approximately so when the scattering properties of the films are widely different. However, even for these cases the advantage of the lo-log representation is considerable, and we feel it promising in this type of work.

Actomyosin, nucleotides, and related substances. In each case, films were slowly evaporated onto a KRS-5 crystal, in an atmosphere of 35% RH at 25°C. Actomyosin films were rinsed with glass-distilled water to remove the KCl and redried (previous work had shown that this procedure did not affect the wave length dependence, but did markedly reduce scattering). It was undesirable to use buffers in these solutions; however, unless otherwise specified, the initial solution was at pH 7. The solutions employed, together with the sources of the materials, are as follows: 1.96% actomyosin in .6N KCl (actomyosin prepared as described by Botts and Morales, '50); 1% (suspension) adenine (Schwarz); 1% ribose (Bios); .02 M sodium tripolyphosphate (Mackay); 0.2 M (buffer) KH_2PO_4, K_2HPO_4 (Merck); 1% adenosine-3-phosphoric acid (Schwarz); .02 M adenosine-5-phosphoric acid (Bischoff); .02 M sodium adenosine diphosphate (Sigma); .02 M sodium adenosine triphosphate (Rohm and Haas); 1% adenosine (Schwarz).

Muscles. For these preparations the medial branch of the *semi tendinosus*[1] of *Rana pipiens* was employed. After overnight equilibration in Ringer's solution at 4°C., the muscles were attached to thin mica rectangles by fine steel wire retractors hooked through the tendons at one end and anchored to the mica at the other. The mica backing served to protect the muscle mechanically and was retained in subsequent operations. The muscles were then carefully dissected into bands about one fiber thick, and the bands were anchored and retracted laterally by fine hooks attached to the mica rectangle. All the foregoing operations were carried out under cool Ringer solution. If the preparation was to serve as a control (i.e. "resting state"), the mica rectangle, with the muscle attached, was rapidly rinsed (for about 5 seconds) in glass distilled water, and then submerged in liquid nitrogen for 30–60 seconds. Isometric preparations exposed to the air were stimulated faradically into a violent tetanus just prior to pouring liquid nitrogen on them; isotonic preparations were similarly treated except that prior to stimulation and freezing the end retractors were undone. In all cases the frozen preparations were rapidly transferred to the lyophilizer (see below).

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[1] The more popular *sartorius* seems to us an especially undesirable muscle to use because of the tough, thick, and rather opaque connective tissue sheath which envelopes the fibers.

The course of the reaction between actomyosin and various substances. Since the experiments were originally designed to reproduce conditions under which the viscosity response of actomyosin was considerable (Dainty et al., '44), the following concentrations were employed in final mixtures: 1.57% actomyosin, .48 M KCl, and .004 M reagent (adenosine triphosphate, etc.). The reactions reported here were carried out at 25°C.; in experiments at 0°C. the reaction rate was somewhat less. Reaction mixtures were stirred gently throughout the period of observation. In all cases very little change occurred after two hours; however, some check observations were always made for a much longer time. In general, there were observed some 10 time points, suitably spaced on the basis of pilot experiments. At a specified time, an aliquot of about 1 cm^3 was withdrawn with a medicine dropper and frozen rapidly on a glass slide which had been over powdered dry ice for at least one-half hour. Slide and sample were then transferred to the lyophilizer. Because our interest was largely in tracing the qualitative course of the reaction no effort was made to set the time of an aliquot to closer than 30 seconds (approximate time which is required for the drop to freeze on the slide); however, it would be a simple matter to do so by using a more refined freezing method.

Lyophilizing, pressing, mounting. Muscles and reaction samples were thoroughly dried for at least 24 hours in a specially-designed large glass chamber, the interior of which was maintained at − 15 to − 20°C., and a pressure of less than .001 mm Hg. They were then withdrawn and allowed to equilibrate with room conditions (25°C.; 35% RH). Both types of specimens were pressed between sheets of stainless steel at about 3000 lb. in^{-2}. The resulting intact films were easily peeled from the steel sheet with forceps and a razor; they were very thin and transparent, and could be mounted over the aperture ($\frac{3}{4}'' \times \frac{1}{8}''$) of a suitable specimen holder. Spectra and the corresponding blanks were always obtained immediately after the films were prepared, although such preparations are no doubt stable for two or three days. Specimens stored for several weeks, however, frequently showed evidence of further reaction in the dry state.

RESULTS AND DISCUSSION

Actomyosin. Spectra of this substance show all the features previously described by several investigators; in particular, the group frequencies of polyamides given by Darmon and Sutherland ('47) are all present at the expected places (fig 1). There are some additional features worthy of mention. The band at 3085^{-1} in all probability corresponds to the 3060 cm^{-1} band described by Ambrose, Elliott, and Temple ('49), and by Darmon and Sutherland ('49). In cyclic amides the latter authors ascribed the 3060 cm^{-1} band to *cis* NHO bonds, and suggested that the 3295 cm^{-1} band of other amides arises from *trans* NHO bonds. Bands at 2906 and 3200 cm^{-1} are also observed in routine preparations; so far as we are aware, these have received no assignment. Our best preparations, i.e., especially thin films on KRS-5 plates, rinsed

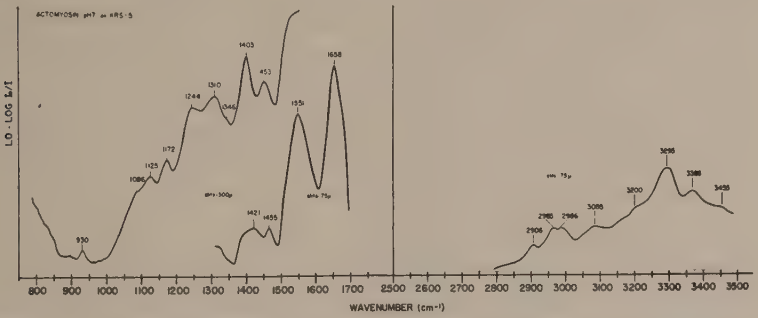

Fig. 1 Absorption spectrum of rabbit actomyosin deposited on a KRS-5 plate at pH 7.

to remove KCl, and examined with narrow slits and an LiF prism, frequently reveal structure on the high frequency side of the 3295 cm^{-1} band, viz. at 3370 cm^{-1} and 3455 cm^{-1}. At lower resolution these appear as a single band midway (around 3420 cm^{-1}) between these two frequencies. Klotz, Griswold, and Gruen ('49) have reported high frequency structure in polylysine and have suggested in that case that it may be due to N-H stretching frequencies in the residues rather than in the backbone. In our case the possibility that this structure may arise from the OH stretching vibrations of serine and threonine residues or from nucleotides strongly bound to the protein (see below) seems equally plausible. Although we have looked repeatedly for evidence of structure in the region where S-H stretching vibrations should appear and although it is a simple matter to demonstrate these bands in cysteine, glutathione, etc., we have not found such structure in actomyosin, at least under conditions in which adjacent (NH, CH) bands were well resolved. It is not feasible with our equipment, to study the S-S vibrations, but the implication is that very little of the myosin sulfhydryl is in the reduced state, at least under the conditions in which we examine it. Another negative finding of possible significance is the failure to alter the high frequency region of the actomyosin spectrum by thorough boiling.[2] Since this observation refers to the solid dry state of the protein, it cannot be considered a decisive fact against assuming that hydrogen bonding plays an important role in heat denaturation, but neither can it be regarded as a fact comforting to the theory. The region between 700 and 1450 cm^{-1} shows a great deal of structure which, as demonstrated by Astbury et al. ('48) and by Klotz et al. ('49) arises largely from the amino acid residues constituting the protein. The region between 800 cm^{-1} and 1100 cm^{-1} is more or less transparent in actomyosin, and, as we shall show below, this is a fortunate circumstance, for it permits unhindered observation of the time course of nucleotide reactions. The exception to this transparency is a band at 930 cm^{-1}, which coincides precisely with a major band (see below) of adenosine diphosphate (ADP) in neutral solution. Taken together with the Buchthal et al. ('49) report that actomyosin contains strongly bound to it adenine, ribose, and phosphate, as well as the earlier suggestions of others (Kalckar, '41; Dainty et al., '49) that certain reactions are best explained by postulating the phosphorylation of actomyosin, it seems plausible to consider tentatively that actomyosin has ADP tightly bound to it. However, there is as yet no evidence to rule out the possibility that the absorption arises from the protein itself.

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[2] From personal communication with Professor I. M. Klotz we understand that he observed no change in the high frequency spectrum of bovine serum albumin as the film was progressively heated by an air stream.

Muscle. Spectra of muscle have been previously reported more or less incidentally (Lenormant, '45; Blout and Mellors, '49). Dubuisson et al. ('43) are apparently the only ones who have investigated this tissue with a view to studying the contraction process (see below). Recently, Barer, Cole and Thompson ('49) reporting on the combined use of the reflecting microscope and the infrared spectroscope, have published a striking spectrum in the regions, 1150 cm^{-1} to 1700 cm^{-1} and 2800 cm^{-1} to 3500 cm^{-1} taken presumably on a single fiber. It is not to detract from the great importance of their method to say that the dissected preparations reported here apparently yield somewhat stronger and better resolved spectra (figs. 2 and 3). In comparing with the spectrum of actomyosin (fig. 1) it is evident that the spectrum of muscle is essentially that of the protein in the region 1200 to 1700 cm^{-1}. Up to and including the 3085 cm^{-1} actomyosin band, the resolution in muscle is likewise about as good as in the protein, and the structure is similar. The higher frequencies, however, are entirely smeared out in muscle, probably by the presence in this tissue of nucleotides and of glycogen. Again, absorption in the region from 980 cm^{-1} to 1200 cm^{-1} of muscle must be ascribed at least in part to substances other than actomyosin, possibly to nucleotides (in particular the band at 980 cm^{-1} may be due to adenylic acid).

Between 860 cm^{-1} and 980 cm^{-1} there is a "transparent" region in which the 930 cm^{-1} can be plainly seen. As suggested above, this band may arise from ADP. It should be mentioned here that, as in the case of myosin films (Ambrose, Elliott, and Temple, '49; Goldstein and Halford, '49), we have found the dichroism of muscle fibers to be very slight or non-existent. This may result from insensitivity for we understand (personal communication) that Professor H. W. Thompson has successed in demonstrating slight dichroism. However, the inference remains that although such bonds (X-H, C = O, etc.) as might give rise to dichroism are insuffici-

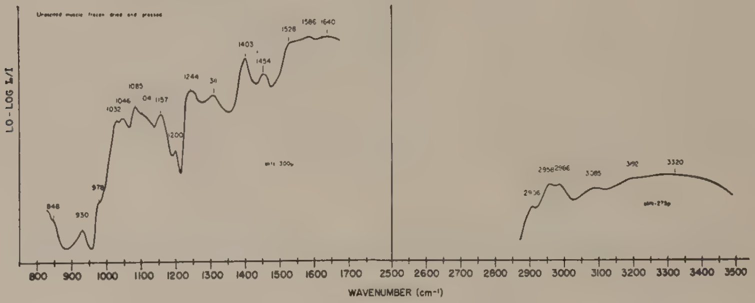

Fig. 2 Absorption spectrum of single fiber band of frog *semitendinosus* rapidly frozen in liquid nitrogen, lyophilized and pressed.

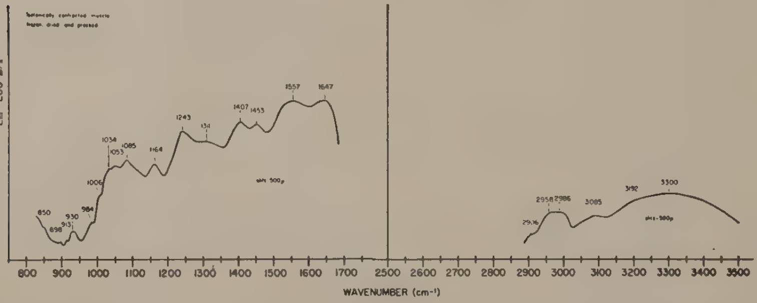

Fig. 3 Absorption spectrum of single fiber band of frog *semitendinosus* rapidly frozen while in isotonic tetanus, lyophilized and pressed.

ently oriented,[3] other structures (presumably of a much greater size in the case of form birefringence) in muscle are well oriented.

Resting muscle vs. isometrically and isotonically contracted muscle. As indicated above, it has been rather difficult for us to obtain spectra of muscle tissue sufficiently reliable to permit *quantitative* comparison between two experimental conditions, say control and contraction. Particularly is this so when just the experimental absorption curves (rather than the lo-log plots) are directly compared. It can be stated categorically, however, that there are no *gross* changes (e.g., disappearance or appearance of major bands) to be observed in comparing resting with either isometrically or isotonically contracted muscle. After examining a rather large number of muscle pairs, relaxed vs. isometrically contracted, we are inclined to the view that in regions where resolution is reliable there are between these two states only the slightest, if any, differences. We have as yet too few experiments to warrant a conclusion in the comparison, relaxed vs. isotonically contracted muscles. There seem to be indications of changes in the region around 1200 cm^{-1} and on either side of 930 cm^{-1} (the 930 cm^{-1} band itself is unchanged). Both of these could conceivably be due to changes in the organic phosphates (figs. 2 and 3). If substantiated by the more thorough investigation now underway, the changes around 1200 cm^{-1} would confirm the earlier work of Dubuisson et al. ('43). The major difference which these authors found between the spectra of actomyosin and of muscle is in the region 980 cm^{-1} to 1200 cm^{-1}, already referred to above. Absorption in 1500–1700 cm^{-1} region and in the hydrogen stretch region is so strong that even using pressed single fiber bands it has not been possible to obtain detail justifying a statement about changes with stimulation. Possibly, the best examination in this region would result from a study of protein-nucleotide mixtures of various proportions.

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[3] Perfect mutually cancelling orientation would likewise preclude dichroism, but such an arrangement is improbable.

Phosphorylated adenosines and related substances. As discussed below, it was found early in this investigation that the reactions of adenosine triphosphate (ATP), adenosine diphosphate (ADP), and the two adenylic acids (A-3-MP and A-5-MP) with actomyosin could be followed best in the region, 800–1300 cm^{-1}. We therefore undertook to study in some detail the 800–1300 cm^{-1} spectra of the various nucleotides and of related substances: adenine, d-ribose, sodium tripolyphosphate ($Na_5P_3O_{10}$), and buffer mixtures of the ordinary sodium phosphates. Blout and Fields ('49) have since published spectra of ribose, adenosine and A-3-MP which with respect to gross features are in agreement with our spectra. In connection with these two and possible future investigations of organic phosphates, we should like to remark parenthetically that in some cases, the portion of the spectrum which arises from the phosphate structure is very sensitive to pH. Thus, in a qualitative analysis for adenylic acid existing, say, in a biological fluid at pH 7, one would be entirely misled by assuming the standard curve to be that obtained by evaporating a film from a solution of adenylic acid in water ($\mathrm{pH} \sim 2.5$) (e.g., fig. 2, p. 337 of Blout and Fields, '49, or figs. 9 and 10 of the present work).

By comparing the spectrum of adenosine (fig. 6) with those of ribose (fig. 5) and adenine (fig. 4), it is possible to correlate many of the bands of adenosine with bands existing in one or the other of its constituents. This is, of course, a theoretically tenuous procedure, but frequently the resemblances are so obvious as to leave no question about the correlation. In turn, the spectra of the adenylic acids may be compared with the spectrum of adenosine, and another set of correlations made. In this last comparison, there appear 5 new structural regions (for A-5-MP) at 880 cm^{-1}, 938 cm^{-1}, 987 cm^{-1}, 1082 cm^{-1}, and 1122 cm^{-1}, which it is reasonable to associate with the introduction of the phosphate radical to the molecule. A summary of the "assignments" made in this fashion is given in table 1.

The structural differences, discussed elsewhere (Tarver and Morales, '50) between the two adenylic acid isomers might be expected to give rise to differences in the infrared spectra of the two substances, as indeed is the case. Among the obvious differences (table 1 and figs. 9 and 10) may be mentioned the comparatively strong bands of A-5-MP at 815 and 838 cm^{-1} and the very weak corresponding bands in A-3-MP, as well as a number of frequency shifts of certain bands: the 938, 1056, 1082, 1122 and 1176 cm^{-1} bands of A-5-MP vs. the corresponding (table 1) bands in A-3-MP.

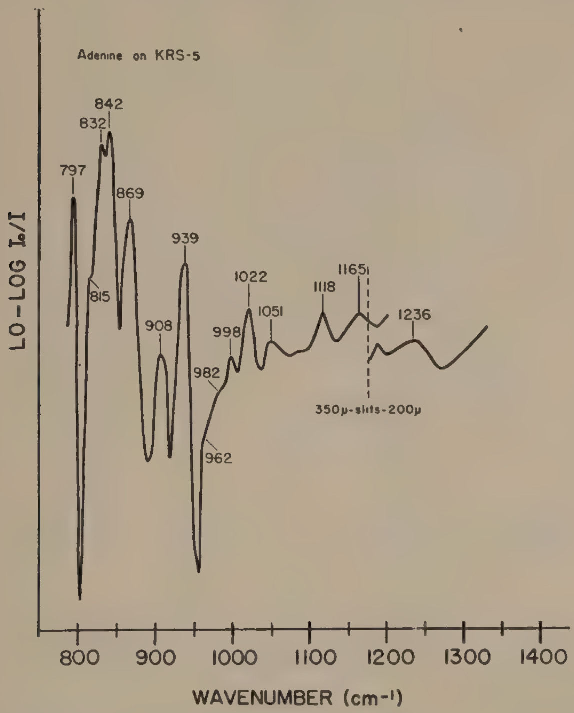

Fig. 4 Absorption of spectrum of adenine deposited on a KRS-5 plate.

While it is easy enough to show that the progressive neutralization of adenylic acid alters the infrared spectrum (figs. 10 and 11) (especially in passing from pH 4 to pH 5), our preliminary work does not permit us unambiguously to trace the fate of the bands originally in the acid. Important obstacles are the increasing accumulation of powerfully scattering salt [4] and ignorance of the ionization constants for the acids. On the basis of preliminary work (not shown here), it appears that in passing from A-5-MP, films evaporated from solutions at pH 2 to those evaporated from solutions at

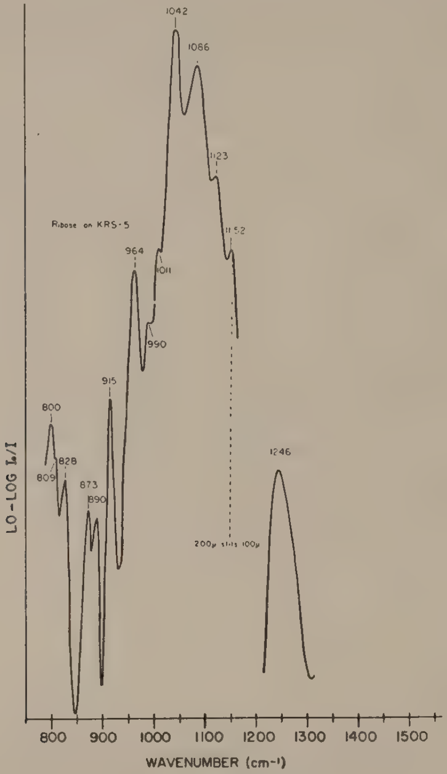

Fig. 5 Absorption spectrum of ribose deposited on a KRS-5 plate.

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[4] The same difficulty was encountered in attempting to acidify solutions of the salts of ADP and ATP. Some spectral changes were induced, but unlike the case of adenylic acid, the major bands seemed to persist, possibly because of the greater acidity of the polyphosphate acids.

pH 7,[5] the following major changes occur: the 1122 cm^{-1} band shifts toward 1100 cm^{-1}, the 1018 cm^{-1} disappears between pH 3 and pH 4 (whether this is a disappearance or a considerable migration, we cannot tell; the observation is in apparent contradiction with the assignment of this band to the ribose sub-unit); the 987 cm^{-1} band grows and becomes the strong 976 cm^{-1} band; the 938 cm^{-1} band appears to weaken and shift toward 918 cm^{-1}.

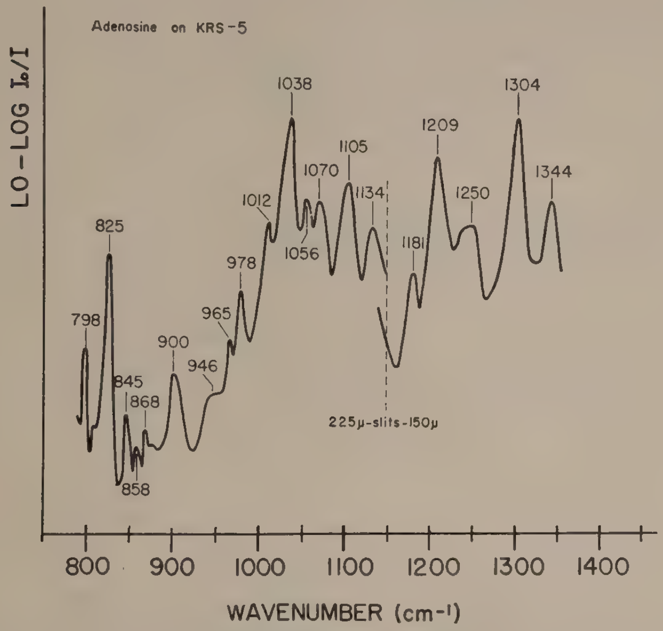

Fig. 6 Absorption spectrum of adenosine deposited on a KRS-5 plate.

In tracing the course of biochemical reactions such as those described below, the required reference spectra are those corresponding to pH 7, i.e., those of the (in this case, sodium) salts of the nucleotides (figs. 7, 8, 11, 12, 13). The major bands observed under these conditions probably arise in part from the phosphate radical, and it is interesting to speculate on their

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[5] Sharp transitions occurred between pH 3 and pH 4, and between pH 6 and pH 7.

TABLE 1

ADENOSINE-5-PHOSPHORIC ACID			ADENOSINE-3-PHOSPHORIC ACID		
WAVE NUMBER (cm^{-1})	INTENSITY OF BAND	PROBABLE SUB-UNIT GIVING RISE TO THIS BAND	WAVE NUMBER (cm^{-1})	INTENSITY OF BAND	PROBABLE SUB-UNIT GIVING RISE TO THIS BAND
785	strong	adenine	793	strong	adenine
815	strong	no assignment made	814	weak	no assignment made
815–830	plateau	ribose 828; adenine 815, 832	824	weak	ribose 828; adenine 815, 832
838	strong	adenine 832, 842	834	weak	adenine 832, 842
860, 870, 880	moderate	phosphate radical	860, 870	moderate	phosphate radical
904	weak	adenine 908; ribose 890, 915	917	strong	adenine 908; ribose 890, 915
938	strong	phosphate radical	951	strong	phosphate radical
987	moderate	phosphate radical	993	moderate	phosphate radical
1018	strong	ribose 1011	1015	strong	ribose 1011
....	.....	....	1030	strong	ribose 1042; adenine 1022
1056	moderate	adenine 1051	1061	strong	adenine 1051
1082	strong	OH bend; phosphate radical; buffer 1080; adenine 1084, 1118; ribose 1086	1095	moderate	phosphate radical; buffer 1080; adenine 1084, 1118; ribose 1086
1122	strong	phosphate radical; polyphosphate; ribose 1123	1126	moderate	phosphate radical
....	.....	....	1140	strong	polyphosphate 1138; ribose 1123
1176	moderate	adenosine 1209	1222	strong	adenosine 1209
1195	moderate	no further sub-division possible	....	.....	....
1248	moderate	adenine 1236; ribose 1246	....	.....	....
1268	weak	no assignment made	1274	weak	no assignment made
1298	weak	adenosine 1304	....	.....	not studied
1325	weak	adenosine 1344	....	.....	not studied

identification. Raman spectra (Herzberg, '45, p. 167) suggested the assignment of bands near 980 cm^{-1} to the symmetrical $P^+(—O)_4$ stretching vibration in the phosphate ion, and 1028 cm^{-1} to the unsymmetrical stretching $P^+(—O^-)_4$ vibration. On the basis of an extensive study of organo-phosphates, Daasch ('50) assigned the stretching frequency of about 1050 cm^{-1} to the P-O-C bond. Thompson (Seminar at National Bureau of Standards, August 25, 1949) assigned the

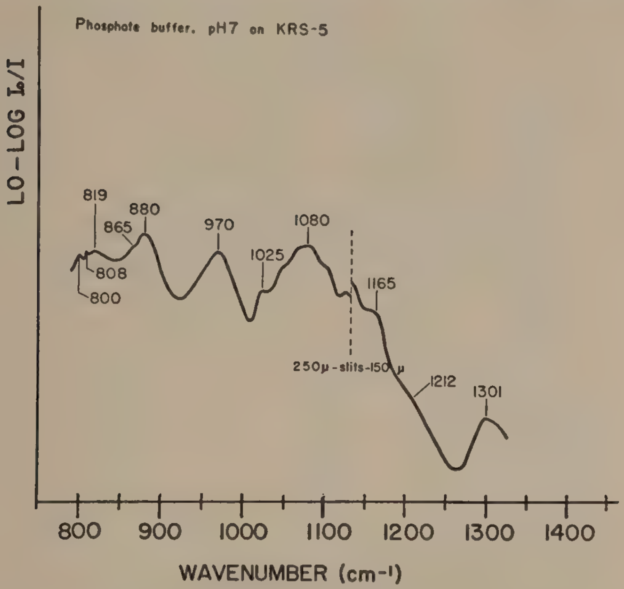

Fig. 7 Absorption spectrum of a phosphate buffer mixture (K_2HPO_4 and KH_2PO_4) deposited on a KRS-5 plate at pH 7.

1240 cm^{-1} region to the P=O stretching vibration. Several workers (Mathieu and Jacques, '42; Simon, '43; Hoffman and Hanwick, '48) have found Raman lines in the region 870–900 cm^{-1} but the assignment seems controversial. Glocker and Campbell assigned 700 cm^{-1} to the symmetrical P-O-P stretching vibration in sodium tripolyphosphate; this frequency lies outside of our present range of study. It is clear that the spectra of the salts used in the present work are not likely to reveal sharp bands at any of these positions; for instance, the deformation of O-H arising from the ribose subunit, would be certain to superimpose on the unsymmetrical $P^+(—O^-)_4$ vibration, as probably also would the P-O-C stretching vibration. Despite these complications, the absorptions observed seem to fall into coarse groups which may be taken as a guide for future work (fig. 14). The presence of a P = O absorption may in a sense be regarded as contradicting the presence of $P^+(—O^-)_4$ vibrations; however, in this connection, it should be noted that except in the case of ATP the "P = O band" is relatively weak, and that mixtures of ionized and unionized phosphate structures are not out of question.

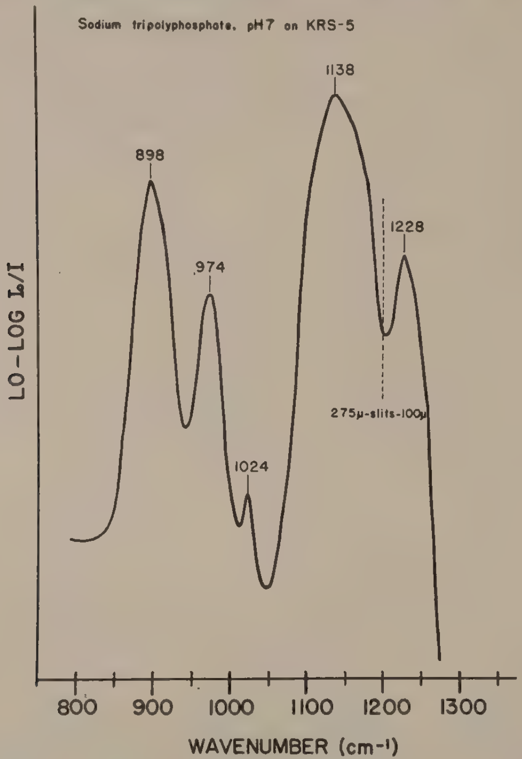

Fig. 8 Absorption spectrum of sodium tripolyphosphate deposited on a KRS-5 plate at pH 7.

Time course of enzymatic dephosphorylation. Entirely apart from the identification of bands in the spectra of the nucleotides, we have found it possible to demonstrate the course of dephosphorylation of adenosine triphosphate by actomyosin and believe it to be the first occasion on which this has been done spectroscopically. The sequences given below are based on some 13 sets of experiments, each using different actomyosin preparations, and there is little doubt about their reproducibility with respect to either frequencies of bands or time of occurrence. It has been shown elsewhere

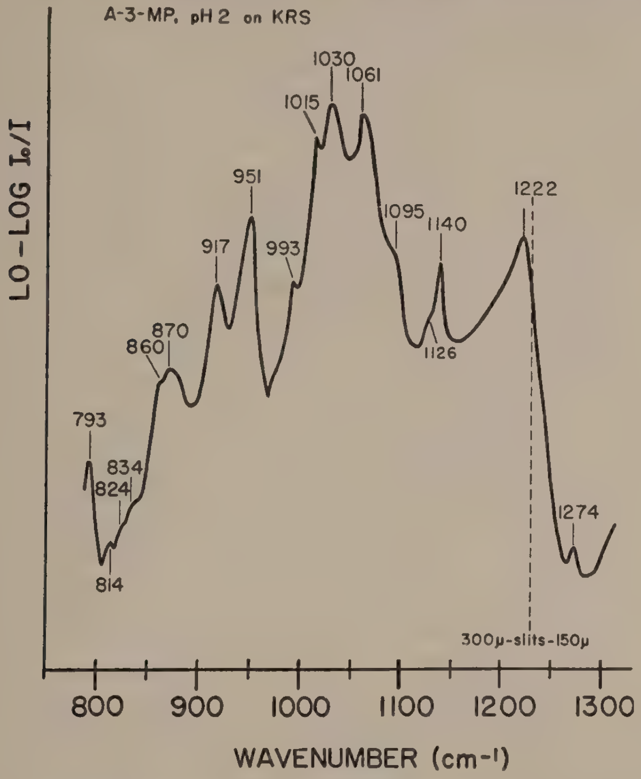

Fig. 9 Absorption spectrum of a commerical preparation of "A-3-MP" deposited on a KRS-5 plate at pH 2. Actually this substance is probably a mixture of NaA-2-MP and NaA-3-MP.

(Tarver and Morales, '50) that using the substances described here, the terminal phosphate of ATP is split off (ATPase action), and either the ADP thus produced is further split, or two molecules of ADP are converted into one molecule of ATP and one of AMP (myokinase action). In the latter case ATP is subject to dephosphorylation, but AMP is not. These reactions all proceed at about pH 7.0. There should, therefore, be a time, just after mixing, when the spectrum of the mixture is the addition spectrum of NaATP and actomyosin; and an intermediate time when the spectrum of the mixture is essentially the addition spectrum of NaADP and actomyosin, and a final time when the spectrum of the reaction mixture is essentially the addition spectrum of con-

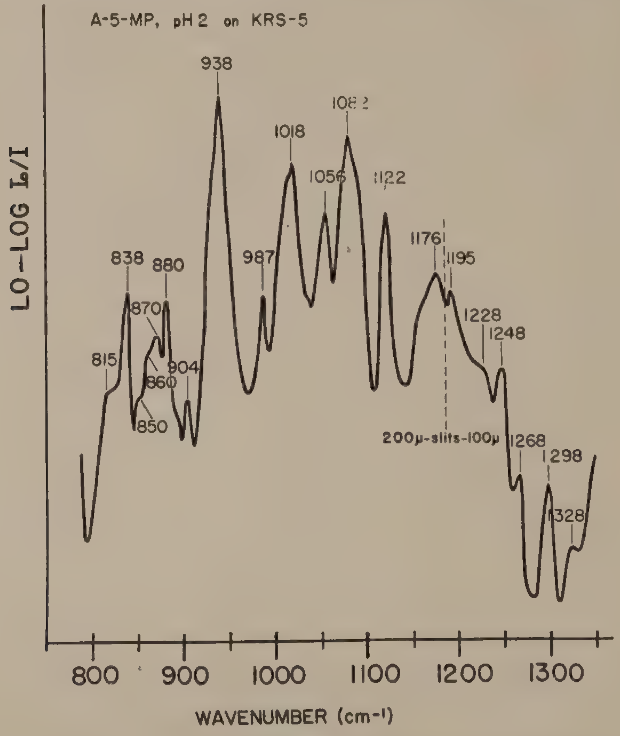

Fig. 10 Absorption spectrum of A-5-MP deposited on a KRS-5 plate at pH 2.

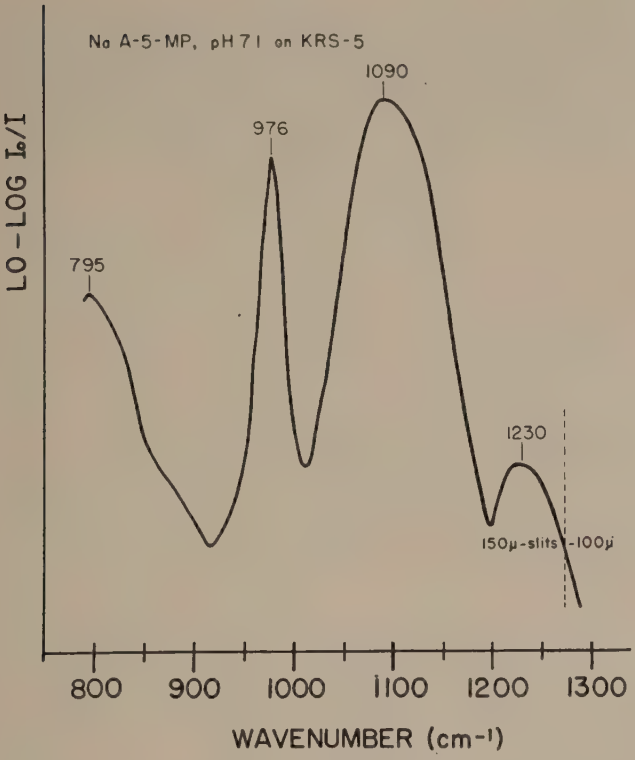

Fig. 11 Absorption spectrum of NaA-5-MP deposited on a KRS-5 plate at pH 7.1.

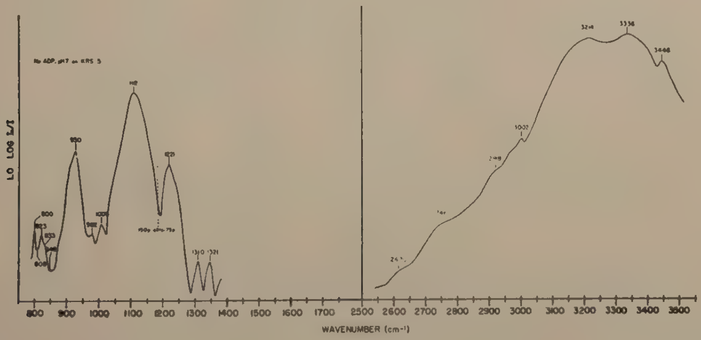

Fig. 12 Absorption spectrum of NaADP deposited on a KRS-5 plate at pH 7.

siderable NaADP, NaAMP, inorganic phosphate, and of actomyosin. Using the concentrations cited above, we found these various mixtures to exist respectively at .33, 13, and 120 (or more) minutes; mixtures frozen at intermediate times showed smooth transitions among these three addition spectra.

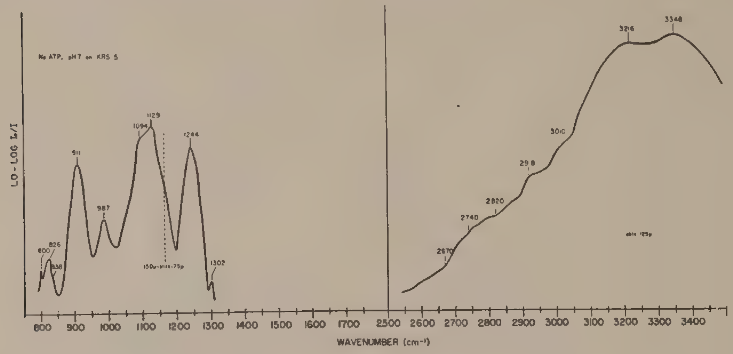

Fig. 13 Absorption spectrum of NaATP deposited on a KRS-5 plate at pH 7.

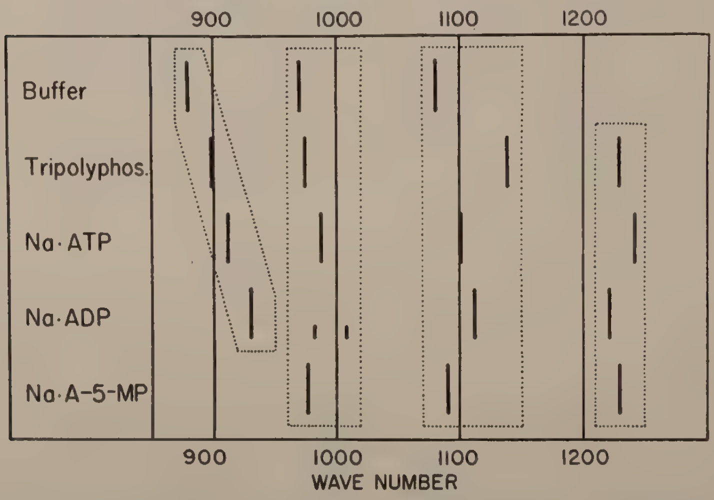

Fig. 14 Certain common frequencies of inorganic and organic phosphates deposited on KRS-5 plates at pH 7. It is probable that the phosphate radical contributes to all 4 absorption groups, but is not alone responsible for these absorptions (see text).

In figure 16, it is clearly seen that the 911 peak of ATP (refer also to the actomyosin control spectrum, fig. 15) present in the .33 minute preparation, has disappeared by 13 minutes and has been replaced by the 930 cm^{-1} peak of ADP. At

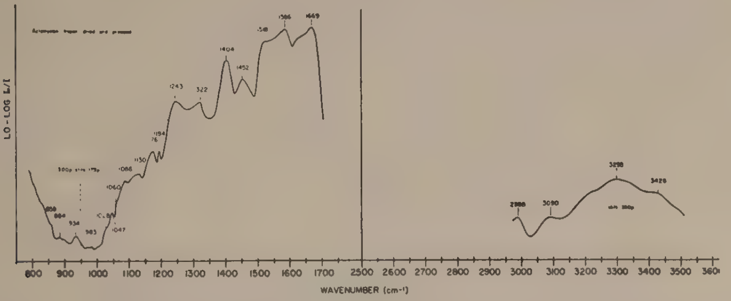

Fig. 15 Absorption spectrum of actomyosin drop, rapidly frozen, lyophilized, and pressed.

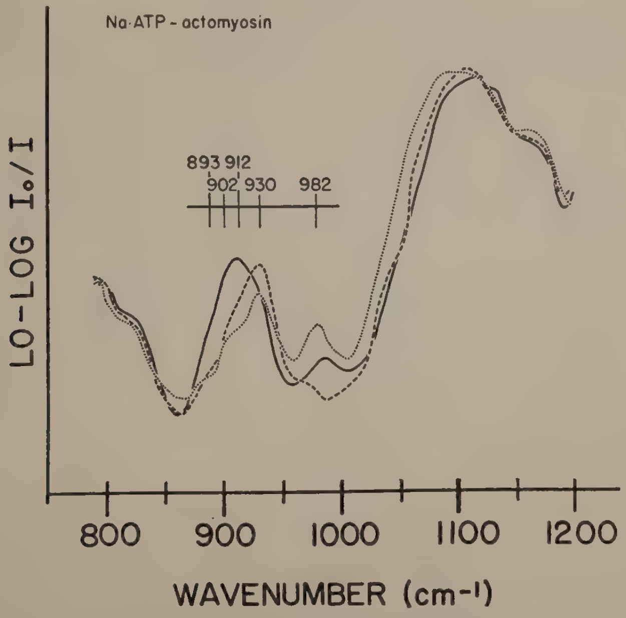

Fig. 16 Absorption spectra of NaATP-actomyosin mixtures, rapidly frozen, lyophilized, and pressed. Mixtures 20 seconds (————————), 13 minutes (– – – – – – –), and 120 minutes (. . . . . . .), after mixing.

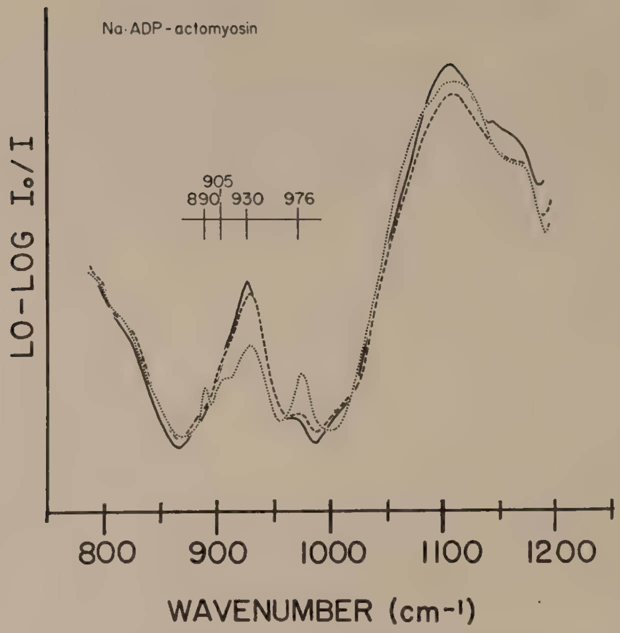

Fig. 17 Absorption spectra of NaADP-actomyosin mixtures, rapidly frozen, lyophilized, and pressed. Mixtures 20 seconds (————————), 13 minutes (- - - - - - -), and 120 minutes (.......) after mixing.

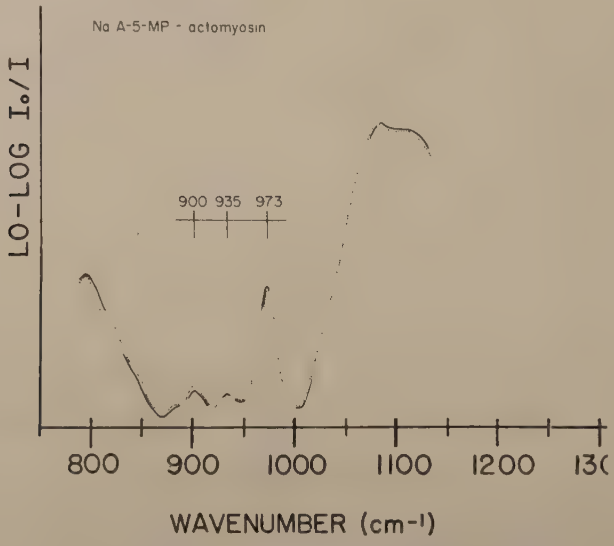

Fig. 18 Absorption spectra of Na-5-AMP-actomyosin mixtures, rapidly frozen, lyophilized, and pressed. Mixtures 20 seconds (————————), and 120 minutes after mixing (. . . . . . .) after mixing.

120 minutes the 930 cm^{-1} peak of ADP has been reduced in size, and the weak 901 cm^{-1} band of AMP (fig. 18) is evident as a shoulder. A new broad band has appeared at about 982 cm^{-1} which is a composite of the 973 cm^{-1} band of AMP (fig. 18) and the 983 cm^{-1} band of phosphate buffer-actomyosin mixtures (fig. 19). When ADP is mixed with actomyosin (fig. 16), the mixture frozen at .33 minute shows only the 930 cm^{-1} band of ADP; there is not much change at 13 minutes, but by 120 minutes the 930 cm^{-1} band of ADP has diminished, and on its low frequency side the 901 cm^{-1} band of AMP has appeared as a shoulder. A strong band has appeared at 976 cm^{-1} which is a composite of the 973 cm^{-1} band of AMP and the 983 cm^{-1} band of phosphate buffer-actomyosin mixtures. Since the inorganic phosphate liberated is in this case smaller than when ATP is used as a

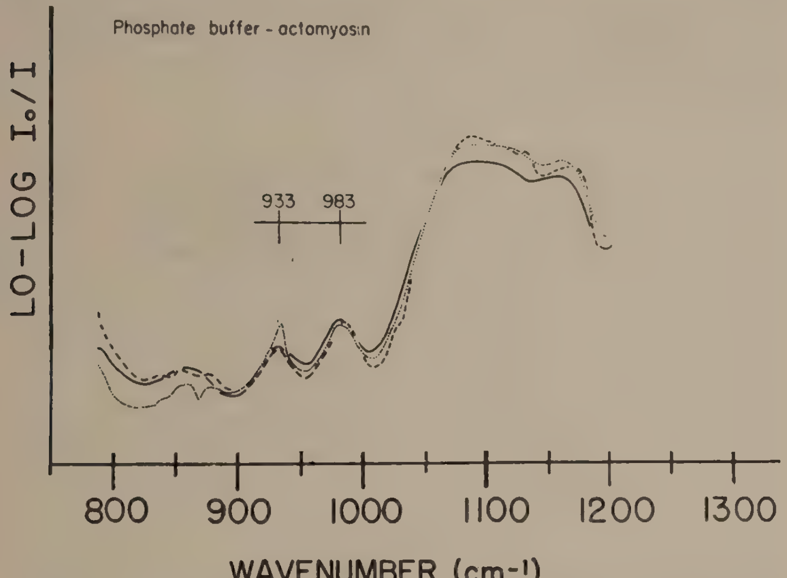

Fig. 19 Absorption spectra of phosphate buffer-actomyosin mixtures, rapidly frozen, lyophilized, and pressed. Mixtures 20 seconds (————); 13 minutes (– – – – – – –), and 120 minutes (. . . . .) after mixing.

substrate, the composite band appears closer to the AMP frequency (973 cm^{-1}). Finally if AMP is mixed with actomyosin, there is no change at all[6] in this spectral region (fig. 18).

The deamination of AMP (demonstrated chemically with myosin by Hermann and Josepovits ('49) and with ultraviolet spectroscopy for this actomyosin by Tarver and Morales ('50) should lead to the appearance of an OH bending vibration around 1080 cm^{-1}, but such a band would certainly be undetectable due to the strong absorption already there.

CONCLUSIONS

It seems evident that the biochemical infrared work on proteins now being pursued in various laboratories will be helpful in explaining many physiological processes. In closing we should like to enumerate certain tentative conclusions and suggestions for future work. In proteins, like actomyosin, rich in hydroxy residues, the absorptions near 3400 cm^{-1} may arise from the stretching vibrations of perturbed OH. Considering the frequently postulated role of SH groups in enzymatic reactions, it is interesting to examine further our tentative result that the SH vibration is not obvious in actomyosin. In the foregoing instances the frequencies at which the OH and SH absorptions should appear are well known, however, such assignments should be checked by demonstrating the disappearance of the absorptions when the protein reacts with group-specific reagents. Conversely, given the assignments, the study of spectral changes on reaction, becomes a method for checking the alleged group-specificity of the reagent for the particular protein. The very low order of dichroism in oriented actomyosin and in muscle itself, taken with the high degree of orientation demonstrable in electron micrographs and in birefringence phenomena, suggests, as remarked above, that high order in the muscle fiber exists at a large scale. Finally, along lines of technique, we should like to suggest that the lo-log representation might in the future facilitate the comparison of spectra of biologically important solids obtained in different laboratories.

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[6] It should be noted, however, that the phosphate buffer band at 970 cm^{-1} (fig. 7) appears to be shifted to about 983 cm^{-1} immediately upon mixing with actomyosin (fig. 19); also, that although A-5-MP alone (fig. 11) has no obvious band around 900 cm^{-1}, such a band does appear immediately upon mixing this reagent with actomyosin (fig. 18).

If the drawbacks of working with dry systems can be tolererated or somehow overcome, as we feel we have done by freezing, etc., it is obvious that infrared spectra hold great advantage over ultraviolet spectra in qualitative analysis for, say, the phosphorylated adenosines. However, perhaps a more interesting application of such spectra is in the elucidation of so-called high energy phosphate bond structure. For this purpose, it will, of course, be necessary to extend the present studies to phospho-pyruvic acid, hexose phosphate, etc., in an effort to find spectral features characteristic of substances in the same energy class.

Our failure to find important changes definitely ascribable to the protein either in the ''hydrogen-stretch'' region or in the ''double bond'' region when actomyosin reacts with the nucleotides or when muscle is excited must, if confirmed by future work, weaken the many theories which imply such changes. By the same token, some support is given to theories which invoke only ''entropic forces'' for contraction and electrostatic forces for extension (see discussion in Botts and Morales, '50), provided the latter have no spectral counterpart in the regions just mentioned. In all schemes, it is necessary to involve some energy yielding reaction as, for example, the ATP-system. As stated above, there is purely analytical evidence that some adenine, ribose, and phosphate are strongly bound to actomyosin, and the absorption of actomyosin at 930 cm^{-1} is so similar to the absorption of NaADP in actomyosin-ADP mixtures that it is interesting to assume that NaADP is indeed bound to the protein; and to speculate on the significance of this arrangement. A plausible hypothesis, elaborated in a stimulating discussion with Dr. John Hutchens, lies in the earlier suggestions of Kalckar

('41) and Dainty et al. ('44) concerning the transfer of phosphate from one molecule to another. One may assume that the terminal phosphate of an ATP molecule in solution is transferred to the second phosphate of an ADP molecule bound to the protein, so that the strucure in solution becomes an ADP molecule, while the structure momentarily on the protein is an ATP molecule. The activation energy of such a transfer would probably be small (for instance such transfers occur easily in "myokinase activity") and the net free energy change would probably also be small. The gain, however, would consist in that the free energy loss in subsequent dephosphorylation might be easily absorbed by the protein as compared with water; in other words, the protein would serve to "stabilize" an ADP-P structure in the ADP + P form, releasing an inorganic P. Thus, an ATP molecule in solution would lose its terminal phosphate only very slowly (as is the case), but an ATP molecule bound to the protein might lose it very rapidly ("ATPase activity"). This hypothesis implies that the "bound-ADP" is attached by the adenine end; this is not out of question since in the deaminase activity of actomyosin the protein certainly binds the NH_3^+ group of the adenine ring, and, crudely speaking, the adenine end seems to offer more "contact area" than does the phosphate chain. The undectability of the ATP spectrum (absorption at 911 cm^{-1}) in muscle might simply result from the fact that in the steady state its concentration is too low. Like all speculations, this hypothesis leaves many facts unexplained; one of these is the myokinase action on the ADP molecules which in the foregoing scheme are rejected from the bound ADP sites. Either these molecules are processed at a totally different site or the "myokinase activity" is merely the further dephosphorylation of a bound ADP molecule; in either case, auxiliary hypotheses, removed from our results, have to be invoked.

Without intending to incriminate the following friends in any shortcomings of this paper, we should like to acknowledge the valuable counsel in the course of this investigation by Mr. L. W. Daasch, Dr. C. Creitz, Dr. F. Smith and Dr. K. S. Cole, and the helpful criticism of the manuscript by Professor G. B. B. M. Sutherland.

Accounts of the foregoing work were presented at the Washington meeting of the Society of General Physiologists, March, 1950, and at the International Physiological Congress, August, 1950.

SUMMARY

1. There is described a freezing, drying, and pressing technique for preparing film specimens from muscles in various stages of contraction and from actomyosin-nucleotide mixtures at various stages of reaction.

2. The lo-log (I_0/I), an absorption function invariant to film thickness or concentration, is discussed and used in all spectra presented. It is suggested that this representation is well suited to the comparison of work from different laboratories.

3. Spectra of the following specimens are presented and several band identifications are made: actomyosin, unexcited frog semi-tendinosus muscle, isotonically contracted muscle, Na-ATP, Na-ADP, A-3-MP, A-5-MP, adenosine, adenine, ribose, Na tripolyphosphate, and phosphate buffer.

4. In addition to absorptions previously reported for actomyosin there are described absorptions in the high frequency hydrogen stretch region which may be due to residue O-H stretch. No changes were observed in the hydrogen stretch region as a result of thermal "denaturation." Under conditions in which N-H and C-H bands were well resolved, no absorption corresponding to S-H stretch was found. The band at 930 cm^{-1} resembles a corresponding absorption in ADP and may arise from ADP tightly bound to the protein.

5. Spectra of muscle appear to be addition spectra of actomyosin and other substances, in particular ADP and A-5-MP. No gross changes occur upon excitation or contraction; however, in comparing unexcited and isotonically contracted

muscle there appear to be spectral differences near 930 cm^{-1} and 1200 cm^{-1}.

6. Spectra of the nucleotides were found to be very sensitive to pH.

7. The time curve of the following reactions was followed spectroscopically: ATP-actomyosin; ADP-actomyosin; A-5-MP-actomyosin; and phosphate buffer -actomyosin. Marked spectroscopic changes were recorded in the dephosphorylation reaction of ATP by actomyosin at .33, 13, and 120 minutes, and in the ADP-actomyosin reaction at .33 and 120 minutes. No changes were evident in the spectra of the other reactions after mixing.

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THE CYTOLYTIC ACTION OF SOME GASTRO-INTESTINAL SECRETIONS AND ENZYMES ON EPITHELIAL CELLS OF THE GASTRIC AND DUODENAL MUCOSA [1,2]

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TWENTY-TWO FIGURES

Opacity and an extremely viscous consistency are gross physical characteristics of one type of mucus produced by the gastric mucosa of man and of animals under a variety of conditions. It has been commonly described as shreds or lumps in secretions collected from pouches or fistulae of dogs, and after excess consumption of alcohol in man.

The presence of leukocytes and epithelial cells or their nuclei has been noted in gastric mucus by Lopez-Suarez ('13), in gastric juice under different conditions by Westphal and Kuckuck ('33), Mulrooney ('40), and Hollander and Feldberg ('41) among others.

In the cat under acute conditions, whitish, opaque, extremely viscous alkaline mucus is regularly produced in response to mild irritants (Grant, '42, '43). Microscopic examination had shown that it consisted of innumerable intact gastric surface

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[2] Preliminary reports of some of this work were presented to the Montreal Physiological Society, December 22, 1942 (Grant, '43), before the Canadian Physiological Society (Grant, '44), and at the September, 1948, meeting of the American Physiological Society (Grant, Grossman and Ivy, '48).

epithelial cells suspended in a clear medium, and that the cells disintegrated leaving only suspended nuclei and granular cytoplasmic remnants when the clear acid component of gastric juice was added to it (Grant, '44).

While testing the possibility that intestinal juice might have a mucolytic effect on gastric mucus it was observed that the cells in the mucus were cytolysed by bile. The disappearance of the opacity and extreme viscosity with the disappearance of the cells affirmed the supposition that these physical properties of the mucus were due to its cellular content (Grant '42), which had been supported by the statistical correlation between increased viscosity, opacity and columnar cell content of mucus from gastric pouches of dogs reported by Hollander, Stein and Lauber ('46).

In addition to the action of bile on the cells in mucus, the action of other gastro-intestinal secretions and enzymes on cells "in situ" and in frozen-dried sections of mucosa will be described.

MATERIAL AND METHODS

Mucus was collected from 24-hour fasted cats under nembutal after the gastric mucosa had been exposed to 30 cm^3 of 1% acetic acid for 30 minutes. The irritant was introduced and removed through a fistula tube 1 inch in diameter which was tied into the greater curvature after the pyloro-duodenal junction had been ligated and a cork drawn into the oesophagus and anchored by a ligature to the oesophagus in the neck to exclude oesophageal and duodenal secretions. The fistula tube was uncorked only for the introduction and removal of irritants and mucus. After the removal of the irritant the cat was kept on its right side.

In the 5 to 6 hour period after exposure to the irritant, 5 to 8 cm^3 of alkaline opaque mucus customarily collected at the mouth of the tube and was carefully removed at intervals. Occasionally the reaction of the mucus changed to acid, falling sometimes to pH 1 to 3 towards the end of the experiment as the clear acid component was added to it. This did not change its gross appearance but on standing a small amount of clear acid component sometimes separated from the opaque fraction. The acid opaque mucus was not mixed with the alkaline opaque collections. Toluol was used as preservative. The mucus was used either at the end of the collection period or kept overnight in the refrigerator before use. Freshly collected gall bladder bile from cat or dog was used. Duodenal juice was collected from dogs with pouches or flaps made from the area of Brunner's gland's, through the kindness of Mr. Delbert Blickenstaff.

The mucus was mixed with the substance being tested, i.e. bile-stained intestinal or duodenal juice, bile and duodenal juice separately, physiological saline, clear acid gastric juice (histamine stimulus), HCl-pepsin solution (0.1% in 0.01 n HCl), 10%–30% sodium dehydrocholate (Na Decholin), 0.2 m Na_2HPO_4, McIlvaine's buffers at pH 2 to 8.5, or with trypsin (0.05 to 0.1%) in the approximate proportions of one part of mucus to one or two parts of other substance.

Tests with bile as well as with one or more of the other substances were made simultaneously on mucus from the same collection period. The substances were mixed in one or more of three ways; (1) as drops on slides, stirred every few minutes and examined microscopically at intervals over a half-hour period, (2) in drop volumes in small stoppered vials which were left overnight at room temperature, (3) in $\frac{1}{2}$ cm³ or 1 cm³ volumes (measured, on account of the viscosity, in graduated centrifuge tubes) in 15 cm³ centrifuge tubes, mixed a few times and left in the ice box overnight. pH was determined electrometrically or with indicator paper.

In some experiments the amount of bile was reduced below the proportion given above, and the amount replaced by either duodenal juice or one of the other substances to test if duodenal juice when mixed with bile had special properties not possessed by the other substances.

The effect of bile on gastric cells "in situ" was studied in 16 experiments by exposing for 8 to 15 minutes a 4 mm area of mucosa beneath a glass tube of this size filled with bile and held against the spot by support from a finger placed on the serosal side. Pressure was just sufficient to prevent the bile from leaking over the adjacent area from the edge of the tube. The area was biopsied within a few minutes after removal of the tube, the biopsy including the exposed spot and also unexposed mucosa outside this spot. The exposed and unexposed areas were separated by a narrow area of superficial damage extending to the neck level of the glands, where the edge of the tube pressed on the mucosa. In addition to controls provided by the surrounding mucosa in this procedure, saline was also used in the same way in 11 experiments. In some cases the resting mucosa of the fasted cats was exposed to bile; in others the test with bile was made 5 to 6 hours after the mucosa had been exposed to acetic acid. Tissues were fixed in 70% alcohol and stained with Mayer's mucicarmine.

Sections of mucosa frozen-dried according to the procedure of Wang and Grossman ('49) were incubated for varying lengths of time with the different secretions, enzymes or control buffers, and subsequently stained with hematoxylin and eosin.

OBSERVATIONS

In table 1 the effect of the different secretions and control solutions on the opacity and viscosity of the mucus in the acid and alkaline states is summarized. Those which formed a clear homogeneous mixture with the mucus were bile-stained intestinal or duodenal juice, bile alone, sodium dehydrocholate and trypsin. For the proportions used the time taken for the maximum effect was approximately one-half hour when the mixture was stirred every few minutes as a smear on a slide; 24 hours when stirred in centrifuge tubes a few times and then left in the ice box for 15 hours; 15 hours when left in vials overnight at room temperature. Besides the ratio of mucus to other substance, the amount of mixing and temperature were probably factors in the time taken for the maximum development of the effect.

The viscosity of the original opaque mucus was too great to be measured in the Ostwald viscosimeter in contrast to the cleared homogeneous mixture which could be so measured.

Substances which did not change the gross physical characteristics of the mucus were physiological saline, clear acid gastric juice secreted in response to histamine, HCl-pepsin solution, dog duodenal pouch juice, McIlvaine's buffers at pH 6, 7.2, and 8.5, and 0.2 m Na_2HPO_4. With these an opaque phase persisted.

TABLE 1

Effect of different substances on the gross physical properties of gastric mucus

SUBSTANCE	TOTAL NUMBER OF TESTS	FINAL STATE OF MIXTURE	
		Clear and homogeneous (number of cases)	Two phases (number of cases)
Bile-stained intestinal or duodenal juice	5	5	
Bile	20 +	20 +	
Duodenal pouch juice	8		8
Saline	10		10
Clear acid gastric juice	11		11
HCl-pepsin solution	6		6
Na decholin	11	11	
McIlvaine's buffer at pH 6, 7.2, 8.5	4		4
0.2m Na_2HPO_4	2		2
Trypsin	4	4	

In table 2 the importance of pH in producing the clear, homogeneous state is shown. In some of these experiments part of the bile was replaced with duodenal juice and the results compared with tests using one of the other substances in place of the duodenal juice. These tests did not show that duodenal juice when present with bile had special properties not possessed by the other substances. However, the pH factor stood out clearly. In the range 7 to 8.4 the clear homogeneous state was produced in all but 2 out of 14 tests. In the two exceptions, numbers 3 and 6, the incomplete effect may be

TABLE 2

Effect of pH in producing the clear homogenous state

TEST NO.	RATIO MUCUS; BILE; DUODENAL JUICE	pH	FINAL STATE OF MIXTURE	RATIO MUCUS; BILE; SALINE (OR 0.2m Na_2HPO_4, OR BUFFER)	pH	FINAL STATE OF MIXTURE
1	4;1;4	8.4	clear, homogeneous	4;1;4 4;1;4	7.5 5.0	clear, homogeneous 2 phases
2	3;1;3 3;1;3	7.7 7.8	clear, homogeneous	3;1;3	6.7	2 phases
3	7.5;1;7.5	7.9	almost completely clear, homogeneous	7.5;1;7.5	7.1	clear, homogeneous
4	3;1;3	7.9	clear, homogeneous	2.6;1;3	7.9(?)	almost completely clear, homogeneous
5	3.8;1;3.8	7.5	clear, homogeneous	3;1;3	7.5	clear, homogeneous
6	6;1;4	7.4	almost completely clear, homogeneous			
7				4;1;4 4;1;4 (0.2 m Na_2HPO_4)	4.4 6.5	2 phases 2 phases
8	4;1;4	7.3	clear, homogeneous	4;1;4 (0.2 m Na_2HPO_4)	7.1	clear, homogeneous
9				1;1;1.5 (buffer) 1;1;1.5 (buffer) 1;1;1.5 (buffer) 1;1;1.5 (buffer)	4 6 7–8 9	2 phases 2 phases clear, homogeneous 2 phases
10	1;1;1 1;0;1 2;1;1 2;0;1	alk alk alk alk	clear, homogeneous 2 phases clear, homogeneous 2 phases			
11				1:1;2 (buffer) 1;1;2 (buffer)	5 7	2 phases clear homogeneous

ascribed to the relatively large amount of mucus in comparison to the bile used. In all 7 tests in the pH range 4 to 6.7 and in one at pH 9, the opaque mucus remained in a separate phase.

In figure 1 (E–F), it can be seen that the gross appearance of alkaline opaque mucus, pH 7–8, (E) is indistinguishable from that of acid opaque mucus (F), pH 2–3, although a small amount of clear secretion tends to separate from the opaque phase in the latter.

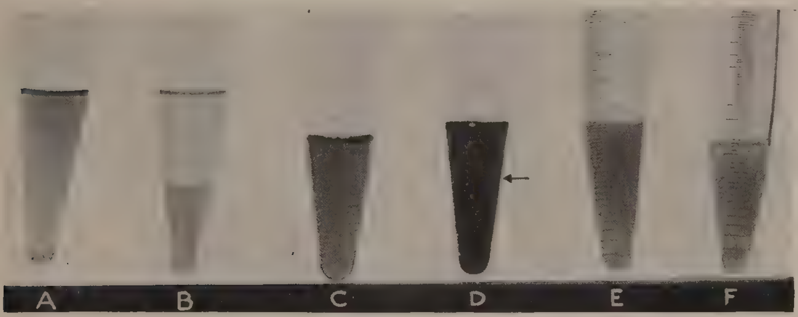

Figure 1

A. Mixture of alkaline opaque mucus, bile and duodenal juice in the proportion of 2;0.3;1. pH 7.8. Homogeneous, clear, one phase.

B. Mixture of alkaline opaque mucus and 0.2 M Na_2HPO_4 in the proportion of 2;1.3. pH 7.8. Two phases.

C. Mixture of acid opaque mucus, bile and duodenal juice in the proportion of 1;0.3;1.5. pH 7.5. One phase.

D. Mixture of acid opaque mucus, bile and saline in the proportion of 1;0.3;1.5. pH 6. Two phases. (Arrow marks surface between phases.)

E. Gross appearance of opaque mucus of pH 7 to 8.

F. Gross appearance of opaque mucus of pH 2 to 3.

Figure 1 (A–D) illustrates the physical appearances of mixtures of mucus with some of the different substances at different pH levels as follows; figure 1 A, a mixture of 2 cm^3 alkaline opaque mucus, 0.3 cm^3 bile and 1 cm^3 duodenal juice at pH 7.8, is clear and homogeneous except for a small amount of debris; figure 1 B, a mixture of 2 cm^3 of the same mucus sample used in figure 1 A, and 1.3 cm^3 0.2 m Na_2HPO_4 at pH

7.8 is in two phases, with the opaque phase reduced in volume from the original after centrifuging; figure 1 C, a mixture of 1 cm^3 acid opaque mucus, 0.3 cm^3 bile and 1.5 cm^3 duodenal juice at pH 7.5, is in one phase; figure 1 D, containing 1 cm^3 of the same mucus as in figure 1 C, 0.3 cm^3 bile, and 1.5 cm^3 saline at pH 6 remains in two phases (the arrow points to the line of separation between the phases).

TABLE 3

Microscopic changes caused by different substances

SUBSTANCE	pH		MICROSCOPIC CHANGE
Bile	8.4		Complete cytolysis.
Na-decholin	Akl.		Complete cytolysis.
Bile stained intestinal or duodenal pouch juice	8.4		Complete cytolysis.
Trypsin	7.5	0.1% 0.05%	Nuclear changes as with duodenal juice.
Duodenal juice	8.4		Nuclei become large, pale, hypochromic. Cell wall may break or show signs of disintegration at this point. Later, nuclear material in cells becomes clumped and scattered, and nuclear membrane is absent.
Natural and artificial gastric juice	1–4		Cytoplasm and cell walls disintegrate. Nuclei shrink and become more basophilic.
Duodenal pouch juice after gastric juice action	7.5		Large pale nuclei and some disintegrated nuclear remnants.
Duodenal pouch juice after gastric juice action	6.9		Two types of nuclei, i.e. large, pale and small, dark.

In table 3 the microscopic changes caused by the different substances are summarized. Those which formed a clear homogeneous mixture with the mucus, i.e. bile, bile-stained duodenal or intestinal juice, sodium dehydrocholate and trypsin caused complete cytolysis of the cells in the alkaline mucus and of the nuclei which are the only formed elements in the acid mucus.

Acid gastric juice, HCl-pepsin solution, and duodenal pouch juice attacked the cells in different ways. With the natural or artificial acid gastric juice, the cytoplasm and cell walls disappeared, as briefly reported previously (Grant, '44), leaving small dark nuclei with sharp-pointed ends, or small more rounded ones with darkly outlined membrane, among granular clumps of residual cytoplasmic material. Numerous refractile spherules accumulated in the disintegrating cytoplasm. Figure 2 (A–C, plate 1) shows this progressive change in the cells in a fresh unstained smear of alkaline mucus exposed to acid gastric juice produced by histamine stimulus. Photomicrographs of the same field were taken at different intervals over a two hour period. The arrows mark two cells in focus in the three pictures. The position of some of the cells has changed in the period during which they were under observation, but the disappearance of the cell bodies and the emergence of the nuclei can be seen over the whole field. It is to be noted that in the original mucus unexposed to acid, but spread on the slide 2 hours before the picture was taken to control the time factor, the nuclei characteristically do not show up (fig. 2 D); but that in figure 2 A contact with the acid gastric juice for 10 minutes, while a suitable field was being found and the picture taken, brought about obvious changes in the cells. Microscopic examination of the opaque phase, which persisted in the presence of the acid for months, showed that it consisted solely of the altered nuclei and granular clumps of material.

Figure 3 A, plate 2, shows the pyknotic nuclei and cytoplasmic remnants in a mucus smear incubated in acid gastric juice for two hours at 37°C. and then stained with methylene blue; figure 3 B shows the same changes in a frozen-dried section of dog gastric mucosa incubated for two hours at 37°C. in 0.1% pepsin in McIlvaine's buffer at pH 2 and then

stained with hematoxylin and eosin. Control sections incubated in buffer were unchanged.

The outstanding microscopic change in the cells exposed to duodenal pouch juice at room temperature, or at 37°C. was the formation of large pale nuclei. In fresh smears the cell walls sometimes first showed signs of disintegration in the vicinity of these swollen nuclei. After some hours, staining with methylene blue showed the nuclear material in some of the cells to be scattered in clumps, and the nuclear membrane appeared to be absent in these cases. These changes did not appear in smears of mucus and saline at room temperature over the same period of time. Incubation of mucus with the duodenal juice for two hours at 37°C. and subsequent staining with methylene blue showed the enlarged nuclei to be hypochromic (fig. 3 C). In controls incubated with saline (fig.3 E) comparatively few cells showed these changes. In sections of frozen-dried gastric mucosa of the dog incubated for two hours at 37°C. in duodenal pouch juice (fig. 3 D) and stained with hematoxylin and eosin the nuclei throughout the mucosa gave no basophilic reaction. Controls, as in figure 3 F, incubated in McIlvaine's buffer at pH 7.8 were normal in staining properties. Duodenal pouch juice acted on the nuclei in frozen-dried intestinal mucosa as it did on gastric mucosa. Trypsin in the crude and crystalline forms in concentrations of 0.05 to 0.1% had an effect similar to duodenal juice; with the higher concentrations cytolysis of the cells in fresh mucus was complete.

When duodenal juice was added to the opaque material left after the action of acid gastric juice, the typical small dark nuclei left by the latter became large and pale, as in duodenal juice alone, when the pH of the final mixture was 7.5. At pH 6.9 the appearance of the smear was intermediate between the appearance in gastric juice alone, and that in duodenal juice alone, i.e. nuclei of both types were present.

The cytolytic effect of bile on the gastric surface cells "in situ" is illustrated in figure 4 (A–D), plate 3. In figure 4 A the mucosa 5½ hours after it had been in contact with 1% acetic acid for one-half hour, was exposed to bile for 8 minutes. In figure 4 B an area from the same section but outside the spot exposed to bile serves as a control. The dark layer on the surface in 4 A stains deeply for mucus and is probably bile or bile mixed with surface mucus. The morphological details of the cells on the free surface are obliterated including the nuclei. The cells lining the crypts are also partly cytolysed and some of the deeper cells show degenerative changes. Blood vessels immediately below the surface are widely dilated. In figure 4 C the mucosa which had been exposed to the bile for 10 minutes was that of the resting 24-hour fasted cat, and in 4 D an area from the same section but unexposed to bile is shown. A wide band of mucicarmine-stainable material covers the area exposed to bile in 4 C and probably consists of a mixture of bile and gastric surface mucus as in 4 A. The intensity of staining is not uniform in the mucus close to the surface cells, light areas adjoining darker ones, suggesting an interaction between the surface mucus and the bile. The nuclei are distorted, shrunken or absent leaving gaps on the free surface in contrast to the normal surface in figure 4 D. Figure 4 E shows the newly-forming surface with low cells containing little or no mucus, and depletion of glandular mucus characteristically seen 5 to 6 hours after exposure to 1% acetic acid. It illustrates the state of the mucosa on which the bile acted in figure 4 A. In contrast to the condition illustrated in 4 E the state of the resting mucosa in the 24-hour fasted cat is shown in figure 4 F. Here the surface cells are tall, well packed together and contain abundant mucus (dark stain). The glandular mucus is also abundant compared to that in 4 E. This represents the state of the mucosa on which bile acted in figure 4 C.

DISCUSSION

The opaque gastric mucus produced by mild irritants may be considered to be of the same origin as the mucus previously described as "visible" (Babkin, '38, '44; Glass, '49).

It is not merely the product of the surface cells but consists of a clear medium, probably partly made up of the secretion of the surface cells, in which are suspended intact surface cells if the reaction is alkaline, or nuclei and cytoplasmic remnants of these cells if the reaction is acid. Under the latter condition the opaque phase, made up of the suspended cellular elements, remains separate from the clear acid fraction. The "native" gastric mucus, which had been considered to be the product of the surface cells, and the mucus "precipitated" from the "native" type by the HCl of the stomach, both classified by Glass ('49) as "visible" are, in our estimation different forms of the same material in alkaline and acid states.

The clear medium in which the cells are suspended probably consists in part of the secretion of the gastric surface cells, the mucicarmine-stainable material which is confined to the upper third of the cell "in situ." It is uncertain if part of the clear medium consists of mucin from the neck and lower parts of the mucosa because its diminution in these areas after irritants may mean that the mucus cells from these areas have taken part in the replacement of shed surface cells rather than that their product has been secreted into the stomach with surface cells (Grant, unpublished).

That the gross physical characteristics of this type of mucus, i.e. its extremely viscous consistency and opacity are due to its cellular content is proved by the fact that only with the substances which cause complete cytolysis, i.e. bile, sodium dehydrocholate, and trypsin does it form a clear homogeneous mixture. Partial cytolysis, on the other hand, caused when the mucus comes in contact with HCl-pepsin either added to it spontaneously in the stomach, or after it had been collected, or exposure to duodenal pouch juice, does not change its gross physical properties.

The viscosity of the clear homogeneous mixture, which in contrast to that of the opaque mucus was sufficiently fluid to be measured by the Ostwald method, was the resultant viscosity of the cytolytic agent, the clear suspension medium, and the products of cytolysis. The final viscosity was still quite high, since probably only that part of the original viscosity caused by the suspended cells had been abolished, and that part of it due to the mucopolysaccharides of the clear medium still remained.

Failure of the bile to form a homogeneous mixture with the mucus when the pH was in the acid range is probably related to the observations of Smith ('14) on the interaction of bile with gastric juice under alkaline and acid conditions. At alkaline reactions, the bile was diluted and spread with the mucus; in the presence of acid it did not mix but reacted with the mucus to form a yellowish-green "precipitated" film. When this film became detached from the surface numerous epithelial cells were found in it. Its insolubility in 0.5% HCl is mentioned, and also its slow solution in excess bile, and a tendency to dissolve in alkaline mucus. He attributed the greater amount of damage produced by bile in the presence of acid, in contrast to that caused by bile alone (confirmed by Yoshitomi, '35, and by Fujioka, '37) to the interference with the protective function of the mucus when it reacted with the bile in the presence of acid. Either the reaction of bile with acid, or the subsequent exposure to acid being the damaging factor. The conclusion of Fiessinger and Palmer ('38) that bile helped to protect the intestinal mucosa by "carpeting" it before the gastric juice arrived, is in line with Smith's view. Smith, however, noted that bile alone caused damage. This may have been due to the cytolytic action of the bile. The presence of epithelial cells which he reported in the film "precipitated" when bile was present with acid suggested to us that part of the precipitate might have been incompletely cytolysed surface cells which remained in a separate phase in the presence of acid. The precipitate which Hammarsten ('11) noted in the course of normal digestion when acid chyme entered the duodenum and mingled with the bile, and which dissolved in excess bile, might also have included incompletely cytolysed surface cells.

Our observation that bile cytolyses the surface cells "in situ" indicates that the living cell is attacked. It is in agreement with Ausbuttel's observation ('39) that ulceration results from bile injected into the walls of the gastric glands; and also with that of Sellards ('09) who reported gastric mucosal ulceration after intraperitoneal injection of bile salts. Still and Carlson ('29) in a study of the motor and secretory activity of the stomach during acute and chronic obstructive jaundice in dogs suggested that bile may cytolyse the acid cells and increase their excitability and acid production.

The reason for cytolysis by bile in the alkaline but not in the acid range in our experiments is not understood. It apparently is not due to precipitation of bile acids since conjugated bile acids are soluble at pH 1. Ponder ('48) suggests that inhibition of the hemolysis caused by bile salts at certain pH levels may be due to changes in the stability of the cells and/or of the lysin. A precipitate settled out when the lysin was brought to the pH at which marked inhibition occurred. The possibility that the isoelectric point of the cells or of one of their constituents might account for the inhibition he regards as only one of many in the complexity of the pH effect. Gordon ('33) reported that the hemolysis caused by bile salts was inhibited at pH 8, and accelerated at pH below 6 — just the reverse of the pH level at which inhibition of cytolysis in our experiments occurred i.e. at levels below 7. This suggests that the mechanism of the effect of pH on cytolysis by bile salts is due to an action on the cells rather than on the bile salts.

The rapidity of cytolysis by bile, especially noticeable in smears on slides at room temperature, suggested a chemical action of a type other than enzymatic. This was supported by the fact that sodium dehydrocholate also was effective. Kettner ('39) who studied the dissolving effect of bile on tissue sections at 37°C. concluded that the action was not enzymatic since heating did not prevent it, and that it was due to bile salts. Goebels and Avery ('29) in a study of chemical changes accompanying lysis of pneumococci by bile found that the organisms could be brought into solution by sodium desoxycholate at 0°C. unaccompanied by any chemical changes which normally accompany true autolysis. Downie, Stent and White ('31) found no parallel between lytic activity of bile on pneumococci and surface tension of the solution; but that the activity was related to the chemical structure of the bile acids.

Differences in the resistance of the gastric surface to bile applied directly in the resting fasted and in the regenerating states were related not only to differences in the amount of mucus present but also to the cellular strength of the surface. Less resistance was offered by the surface 5 to 6 hours after 1% acetic acid than by the resting mucosa in the fasted state. In the former case many areas of the surface are made up of flat immature cells (Grant, '45) replacing the mature cells shed in response to the irritant. In contrast to this condition, the resting mucosa of the fasted state consists of tall columnar closely packed surface cells. The lessened resistance to damage by bile and acid 3 to 6 hours after feeding compared to that presented by the fasting stomach which Smith ('14) attributed to scarcity of mucus while gastric juice was still abundant may have been partly due to the difference in cellular strength of the surface, for we have some evidence that the loss of these cells is considerable (Grant, '44) in the early stages of digestion. Also Becamp (1882), though presenting no supporting evidence, believed that the constant formation of new cells to take the place of those disappearing through usage explained the resistance of the gastric mucosa to peptic digestion. Besredka ('19) explained the lowered resistance of rabbits to parathyroid B bacilli when bile was given orally preceding the bacilli, to the desquamation of the intestinal mucosa which helped the absorption of the virus, although he presented no evidence for desquamation.

The strikingly different effect produced on the nucleus by natural or artificial acid gastric juice or pepsin on the one hand, and by duodenal pouch juice and trypsin on the other requires consideration of substrates on which these enzymes may possibly act. The darkening and increased basophilia seen in the nuclei in fresh unstained and in fixed stained cells respectively, and the shrinkage in both cases under the influence of acid gastric juice or pepsin solution suggests a concentration of nucleic acids of the chromatin. Basophilia which is determined by the nucleic acids of chromatin is not dependent on conjugation of the nucleic acids with protein (Dempsey and Wislocki, '46) so that a destruction of the protein fraction of the desoxyribonucleoprotein of the nuclear chromatin could explain the increase in basophilia and decrease in volume. In this connection Mathews' ('39) description of the changes in lower forms when nuclear sap, present in these forms in addition to chromatin, mixes with cytoplasm is of interest. Carbon dioxide is produced as respiration is stimulated, the chromatin shrinks, and its avidity for basic dyes increases "as if considerable amount of protein had been digested or separated from it." The refractile spherules which rapidly accumulated in the disintegrating cytoplasm under the influence of the acid-pepsin and also of duodenal juice and trypsin in our experiments may have had an origin similar to the spherules in the cytoplasm of Asterias vulgaris eggs undergoing disintegrative changes in the presence of nuclear sap described by Mathews. However the cytoplasm may be destroyed by peptic activity rather than by autolytic enzymes liberated from the nucleus. It is of interest that in Miescher's study (1871) which led to the discovery of nuclein, nuclei were isolated by digesting the cytoplasm with HCl-pepsin. Mathews ('39) states that the protamine present in nuclei of some lower forms is not digested by HCl-pepsin, but is digested by trypsin.

The decrease in nuclear basophilia which is the first outstanding effect produced by duodenal pouch juice and by trypsin in fresh and in fixed cells may indicate a loss of desoxyribonucleic acid from the nuclei. A phosphoesterase from calf intestinal muscle hydrolyses desoxyribonucleic acid completely after treatment with nuclease from the pancreas, and 70% hydrolysis occurs without such pre-treatment (Zittle, '47). These enzymes may have taken part in the cellular changes we observed. With the simultaneous swelling of the nuclei the decrease in staining reaction might be either a secondary dilution effect or primary and lead to the swelling. Ramond ('04) reported karyolytic changes i.e. enlarged nuclei and less selective staining reactions in intestinal epithelial cells as they passed in the chyme along the digestive tract. What part these cytolytic changes in the gastric and duodenal cells brought about by known digestive enzymes may play in the processes of digestion and absorption of food remains to be clarified.

SUMMARY AND CONCLUSIONS

A study is described of the action of some of the gastrointestinal secretions and enzymes on (a) gastric surface epithelial cells suspended in alkaline opaque mucus secreted by the cat's gastric mucosa in response to mild irritants; (b) on the nuclei suspended in opaque mucus which had been exposed to clear acid gastric juice secreted in response to histamine, or to HCl-pepsin solution; (c) on mucosal cells "in situ;" (d) on frozen dried sections of mucosa.

Bile completely cytolysed the suspended cellular elements in the mucus, originally either acid or alkaline, when the pH of the final mixture of bile and mucus was 7 to approximately 8.5. Simultaneously the opacity and extremely viscous state of the mucus disappeared and a clear, homogeneous mixture was formed. The opacity and extremely viscous state of the mucus apparently are caused by the cellular elements suspended in it. Sodium dehydrocholate and trypsin in certain concentrations also caused complete cytolysis.

The gastric surface cells "in situ" in areas of mucosa which had been exposed to bile for short periods of time began to disintegrate. Degenerative changes were more easily produced in regenerating surfaces, characterized by flat immature cells containing little or no mucus, than in the resting surface of the fasted cat, made up of tall closely packed cells containing abundant mucus.

Gastric juice of the histamine-stimulus type, and HCl-pepsin solution destroyed the cytoplasm of the cells suspended in the alkaline mucus. The nuclei remained suspended but were shrunken in size and appeared darker than they were in the intact cells in fresh mucus smears. The disintegrated cytoplasm appeared as granular clumps with a yellowish tinge. In spite of the microscopic changes the gross physical properties of the mucus in the presence of acid, i.e. its opacity and viscous state were indistinguishable from the mucus in the original alkaline state. The mucus containing the suspended nuclei and granular cytoplasmic remnants remained in a separate phase from the clear acid component at pH 1–3 for many months. In frozen-dried sections of mucosa incubated in buffer-pepsin solution and subsequently stained with hematoxylin and eosin, disintegration of the cytoplasm, shrinkage of nuclei, and increased basophilia occurred in all mucosal cells.

The outstanding effect of duodenal pouch juice was on the nuclei which became swollen and pale in fresh unstained smears of mucus, and hypochromic when these were stained with methylene blue. The nuclei of all mucosal cells in frozen-dried sections incubated in duodenal juice lost their basophilic reaction or completely disappeared.

The cytolytic action of trypsin on the cells in mucus and in frozen-dried sections was complete in the higher concentrations and over the longer periods of time. In weaker concentrations and over shorter periods nuclear changes similar to those caused by duodenal pouch juice occurred.

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PLATE 1

EXPLANATION OF FIGURES

2 Unstained smear of fresh alkaline opaque mucus at progressive intervals after the addition of histamine-stimulated clear acid gastric juice. Arrows point to two cells in focus.

A. Ten minutes after exposure to acid juice.

B. Forty minutes after exposure to acid juice.

C. Seventy minutes after exposure to acid juice.

D. Control smear of mucus unexposed to acid but prepared at the start of the test in order to rule out the time factor.

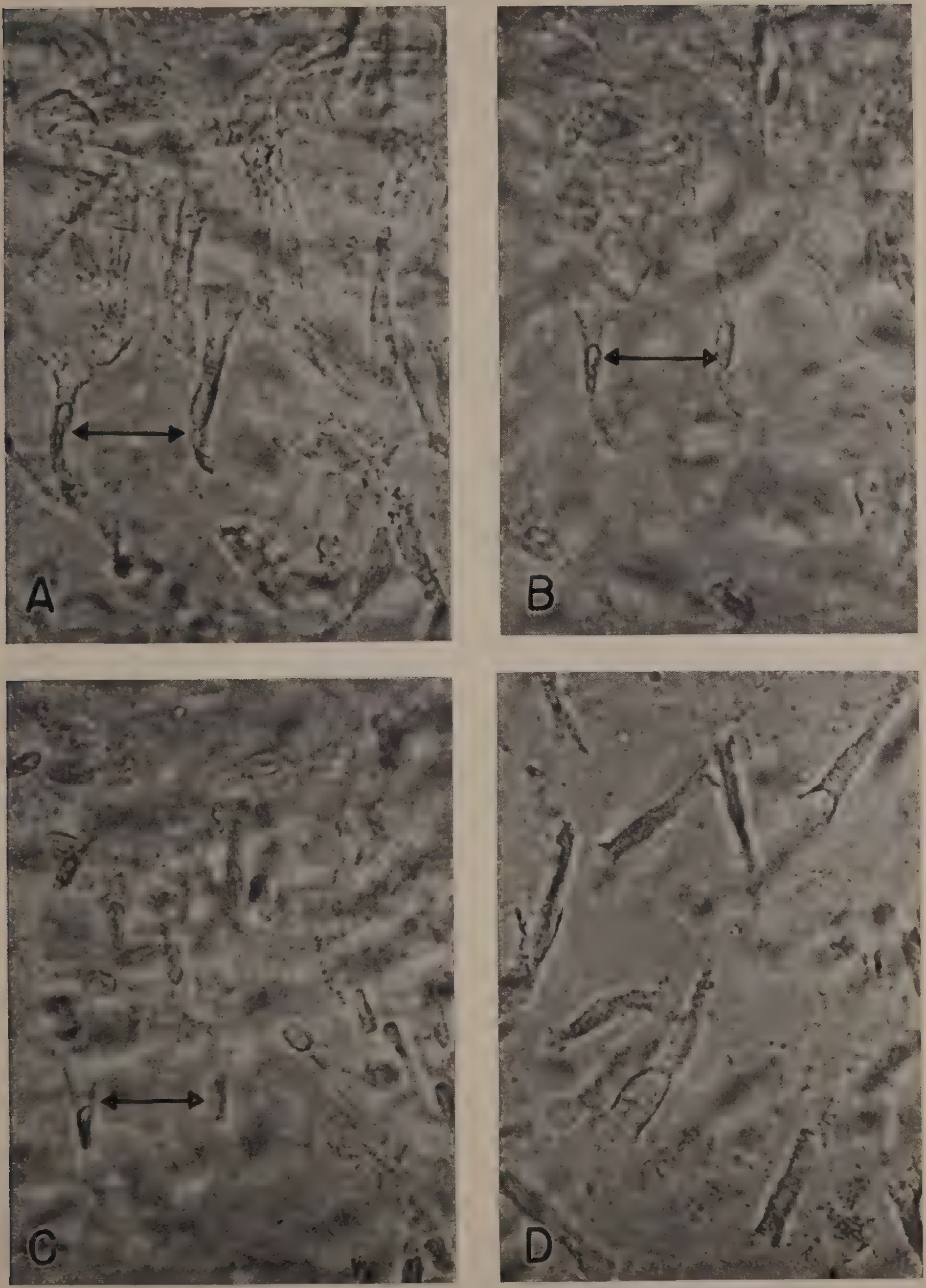

A
B
C
D

PLATE 2

EXPLANATION OF FIGURES

3 Alkaline opaque mucus smears or frozen-dried sections of gastric mucosa incubated 2 hours at 37°C. in different agents. Smears subsequently stained with methylene blue; sections with hematoxylin and eosin.

A. Mucus smear incubated in acid gastric juice. Cytoplasm disintegrated; nuclei pyknotic and dark. × 600.

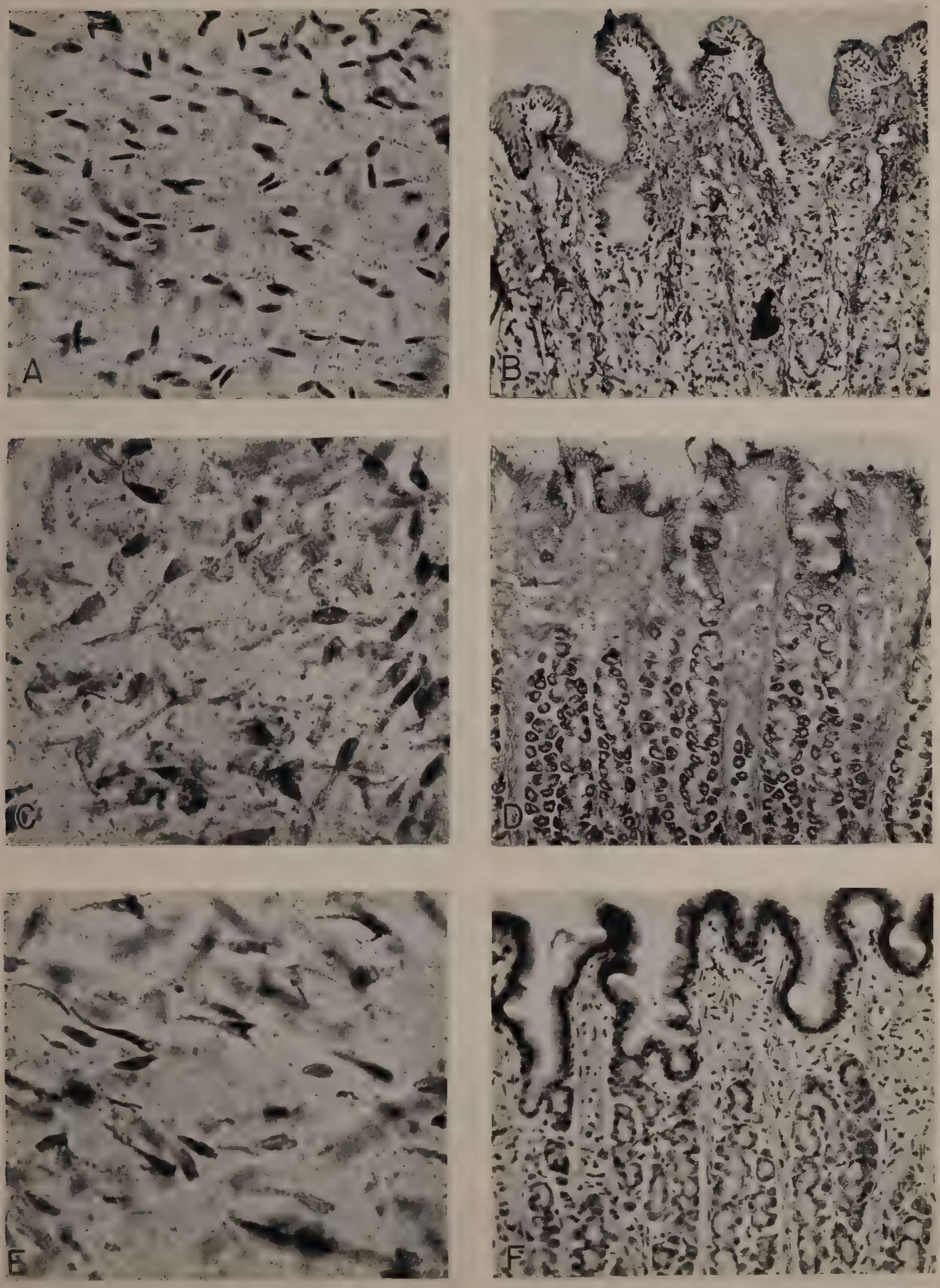
B. Frozen-dried section incubated in pepsin (0.1%). Nucleic pyknotic dark. × 200.

C. Mucus smear incubated in duodenal pouch juice. Nuclei pale swollen. × 600.

D. Frozen-dried section incubated in duodenal pouch juice. Nuclei pale, swollen or completely disintegrated. × 200.

E. Mucus smear incubated in saline. Control for A and C. Less marked changes in the nuclei. × 600.

F. Frozen-dried section incubated in buffer at pH 7.4. No marked cellular changes. × 200.


A
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C
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PLATE 3

EXPLANATION OF FIGURES

4 Gastric surface cells exposed "in situ" to bile. Fixative, 70% alcohol; mucicarmine stain. Cat.

A. Ten minute exposure to bile, 5½ hours after one-half hour exposure to 1% acetic acid. (Exposure to acetic acid lowers the strength of the surface by reducing the cells numerically. The surface at this time is typically covered with low cells containing little or no mucus, as in E). × 430.

B. Control area from the same section from which A was taken but unexposed to bile. × 430.

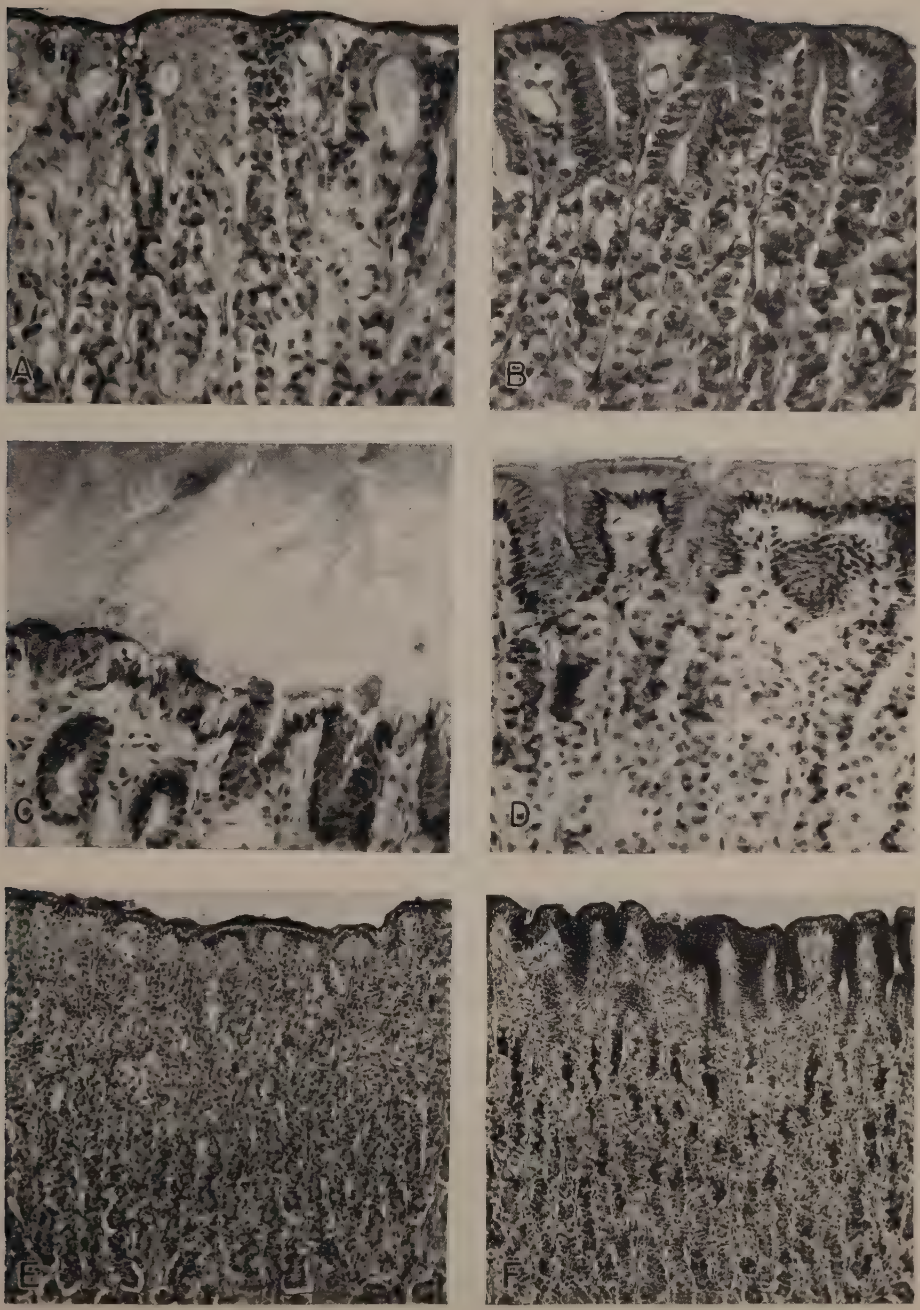
C. Eight minute exposure to bile (resting mucosa in fasted state). × 430. Note thick layer of mucus covering area.

D. Control area from the same section from which C was taken but unexposed to bile. × 430.

E. Typical appearance of regenerating surface 5 to 6 hours after removal of cells by irritant. Low surface cells contain little mucus. Glandular mucus scant. × about 120.

F. Typical appearance of mucosa in 24-hour fasted cat. Surface cells tall, closely packed and containing abundant mucus. Glandular mucus (dark) abundant. × about 120.

(E and F illustrate states of surface on which bile acted in A and C respectively.


A
B
C
D
E
F

RADIATION EFFECTS ON LIVER GLYCOGEN IN THE RAT

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FOUR FIGURES

Irradiation has been shown to result in a decrease in the food intake of rats together with an impairment of gastric digestion (Ely and Ross, '47). From these findings it would be expected that the amount of hepatic glycogen would decrease after irradiation. The results of the present study however, indicate that the amount of hepatic glycogen does not decrease after irradiation. The amount of hepatic glycogen, on the contrary, was found to increase when fasted animals were irradiated. This increase in the amount of hepatic glycogen apparently was not due to the irradiation of the liver or of the adrenals, for it occurred even when these portions of the body were protected by lead shielding.

MATERIALS AND METHODS

Eight hundred and thirty male white rats of the Wistar strain were allowed Fox Food Blox *ad libitum* for a number of weeks prior to any treatment. Each group in each test consisted of 10 or more rats.

Rats approximately 150 gm in weight were irradiated in groups of 5 with x-rays or with neutrons. The x-ray apparatus, Maximar 250 Kv, was operated at 200 kvp, 15 ma with 3 mm of aluminum (inherent) and with ½ mm copper filter. The neutron irradiation was given in the manner described by Enns, Terrill, and Garner ('47). Anesthesia was accomplished by the intraperitoneal injection of Nembutal. For protection from x-irradiation, a sheet of lead 5.5 mm in thickness was placed directly over the region of rat to be protected. The lead sheet was bent so that the sides of the rat were protected from the x-rays as well as the portions facing the x-ray tube.

The rats were decapitated, the adrenals and livers weighed and small pieces about 3 × 5 × 3 mm of the left lateral lobe of the liver were removed. These pieces were placed in Carnoy's fixative (ethyl alcohol-acetic acid 3-1) within one-half minute after decapitation.

Composite blocks were prepared containing one piece of liver from a rat of each group in each experiment. This procedure insured uniform thickness of sections, 6 μ, and identical subsequent treatment.

The glycogen in the liver sections was demonstrated cytologically by the Hotchkiss ('48) modification of the Bauer-Schiff technique. Glycogen was also demonstrated in certain of the sections by Best's carmine method and Gomori's ('46) methenamine technique. Control sections were digested with saliva at 40°C. for one-half hour before hydrolysis and staining.

EXPERIMENTAL

The food intake of non-irradiated rats was restricted for experimental periods of 24, 48, and 72 hours respectively to the amount consumed by rats 24, 48, and 72 hours after radiation with 100 n. Little glycogen was found in the liver sections of the non-irradiated rats which had had their food restricted. Large deposits of glycogen, however, were found in all the livers of the rats which had been irradiated, similar to the large deposits found in the livers of the non-irradiated rats which had had food *ad libitum*.

Rats were depleted of liver glycogen by fasting for 24 hours before radiation, then given a dose of 100 n and the fasting was continued for 24 more hours. Large deposits of glycogen were found in the livers of fasted irradiated rats similar to those in the livers of the non-irradiated rats which had

had food *ad libitum* but only traces were found in the livers of non-irradiated rats fasted for 24 and 48 hours respectively (see figs. 1–4).

In order to determine the time of appearance and the duration of the presence of glycogen in the liver after radiation,

TABLE 1

Liver glycogen in rats after various doses of neutron and x-radiation and after different periods of fasting

Total	FASTED		RADIATION DOSE	NO. OF RATS	GLYCOGEN
	Before radiation	After radiation			
hours	*hours*	*hours*			
0 (food *ad libitum*)			0	55	Large deposits
24	. .	. .	0	25	Trace
25	24	1	100 n	10	Large deposits in 7
27, 30, 40	24	3, 6, 16	100 n	30	Large deposits
48, 72, 96	. .	. .	0	45	Trace
48, 72, 96	24	24, 48, 72	100 n	30	Large deposits
24	. .	. .	0	10	Trace
25	24	1	500 r	10	Large deposits in 7
27, 30, 42, 48	24	3, 6, 18, 24	500 r	40	Large deposits
24	. .	. .	0	25	Trace
48	24	24	50 r	15	Large deposits in 9
48	24	24	100 r	15	Large deposits in 10
48	24	24	200 r	15	Large deposits in 12
48	24	24	300 r	15	Large deposits
48	24	24	400 r	15	Large deposits
48	. .	. .	0	15	Trace
48	24	24	10 n	15	Large deposits in 12
48	24	24	25 n	15	Large deposits
48	24	24	50 n	15	Large deposits
48	24	24	100 n	15	Large deposits

fasted rats were irradiated and sacrificed after various intervals of continued fasting (see table 1). The shortest period of time after radiation after which glycogen was found in the livers of all the neutron or x-irradiated rats was three hours. Three days after neutron radiation (a total fasting period of 96 hours) large amounts of hepatic glycogen were still observed.

The smallest doses of neutron and x-radiation respectively which resulted in an observable increase in the glycogen content of the livers of all fasted rats, were found to be 25 n and 300 r (see table 1).

When the liver region (extending from the axilla region to one-half inch posterior to the xyphoid cartilage) of the anesthetized rats fasted 24 hours before and after radiation was protected with lead shielding and given an x-ray dose of 1000 r, deposition of glycogen was not prevented. Nor did protection of the lower half of the rat (from the posterior aspect of the liver) or the anterior half (from the xyphoid cartilage forward, which of course protected the pituitary gland) prevent deposition. As previously found, the livers of rats which had been fasted for 24 and 48 hours contained only traces of glycogen and large deposits were found in the livers of rats which were on an *ad libitum* diet. The deposition of glycogen in the livers of the fasted, fed or irradiated rats was identical with that shown in figures 1–4. No alteration was observed in the amount or site of liver glycogen in the anesthetized rats.

Insulin was administered in doses, 4 units per kilogram rat weight, to rats on an *ad libitum* diet. Slightly increased amounts of glycogen were observed. When insulin was administered to fasting rats, the already low level of liver glycogen was still further reduced. When administered to fasting irradiated rats, a slightly smaller amount of glycogen appeared to be present in the liver cells than in the cells of the fasted irradiated rats (see table 2) without insulin.

When adrenalin was administered intraperitoneally in doses of 0.5 mg per kilogram of rat weight, to fasted irradiated rats, only traces of glycogen were found, while in fasted irradiated rats without adrenalin, large deposits were noted (see table 2).

Weight of livers and adrenals. The weight of the livers as well as the total body weight of the rat decreased when the rats were fasted for 24 and 48 hours. The weight of the liver per 100 gm of rat weight was also lower than that of livers from rats fed *ad libitum*. The weight of the livers of fasted irradiated rats decreased only slightly compared to the weight of livers from rats fed *ad libitum* but was 31% greater than the weight of livers from rats fasted the same period of time (see table 3). The weight of the livers of rats fasted for 12 hours, irradiated with a dose of 1000 r (adrenals protected by lead shielding) and then fasted for another 72 hours was 46% greater than that of the livers of rats fasted for a total of 84 hours (see table 4).

TABLE 2

Influence of insulin and adrenalin on liver glycogen of irradiated rats

	FASTED					
Total	Before radiation	After radiation	RADIATION DOSE	SUBSTANCE INJECTED	TIME OF INJECTION BEFORE KILLING	GLYCOGEN
hours	*hours*	*hours*			*hours*	
0 (food *ad libitum*)			0	0	0	Large deposits
0 (food *ad libitum*)			0	Insulin [1]	3	Large deposits
48	..	..	0	0	0	Trace
48	..	..	0	Insulin [1]	3	Minute trace
48	24	24	100 n	0	0	Large deposits
48	24	24	100 n	Insulin [1]	3	Large deposits
0 (food *ad libitum*)			0	0	0	Large deposits
0 (food *ad libitum*)			0	Adrenalin [2]	1½	Large deposits
48	..	..	0	0	0	Trace
48	..	..	0	Adrenalin [2]	1½	Trace
48	24	24	100 n	0	0	Large deposits
48	24	24	100 n	Adrenalin [2]	1½	Trace

[1] Booster shot 20 minutes before killing.

[2] Booster shot ½ hour before killing.

Because of the relationship between adrenal cortical hormones and glycogen deposition, and because of the reported (Patt, Swift, Tyree, and John, '47) changes in adrenal weight following whole body x-radiation, weight determinations

TABLE 3

Changes in liver weight of rats 24 hours after 1000 r whole body x-radiation

	AVERAGE RAT WEIGHT (*final*)	AVERAGE LIVER WEIGHT	LIVER WEIGHT / RAT WEIGHT
	gm.	*gm.*	%
Unirradiated, food *ad libitum*	176.5	8.47	4.80
Unirradiated, fasted 24 hours	160.5	6.03	3.76
Unirradiated, fasted 48 hours	148.4	5.03	3.39
Irradiated (1000 r) fasted 24 hours before and 24 hours after radiation	154.4	6.88	4.45

TABLE 4

Weights of adrenal and liver before and after 1000 r x-radiation

TREATMENT	FASTED			RAT WEIGHT	ORGAN WEIGHT / RAT WEIGHT	
	Total	Before Radiation	After Radiation		Adrenal	Liver
	hours	*hours*	*hours*	*gm*	%	%
Unirradiated (Food *ad libitum*)	..	..	..	225.9	0.0062	4.73
Unirradiated[1] (Food *ad libitum*)	..	..	..	226.7	0.0068	4.66
Unirradiated (Fasted)	84	..	..	159.3	0.0095	3.82
Unirradiated[1] (Fasted)	84	..	..	160.7	0.0097	3.12
Irradiated[1] (Fasted)	84	12	72	161.0	0.0144	4.57
Adrenal region protected by lead shielding						

[1] These groups were under Nembutal anesthesia for about 1 hour, 72 hours before sacrificing.

were made of adrenals from irradiated rats whose adrenals had been protected by lead shielding. An increase in adrenal weight was found in fasted irradiated rats (see table 4).

DISCUSSION

The fact that glycogen is stored during periods of starvation-and-irradiation may indicate that the utilization of carbohydrate is affected by radiation. If the effect of radiation were directly on the liver, then protection of the liver by lead shielding would prevent glycogen deposition and the increase in the weight of the liver. Glycogen deposition in the liver after irradiation thus appears to be secondary to some more deep-seated effect. If one particular organ only had been involved, then protection of that organ by lead would have prevented glycogen deposition.

It might be assumed that the adrenal gland has an important role in the hepatic glycogen deposition found after radiation. Long, Katzin, and Fry ('40) have shown that the introduction of an excess of adrenal cortical hormones into the animal resulted in an increase in the amount of glycogen even in the fasting animal. There is, however, no proof at present that the increased adrenal size, implying an increased demand and production of adrenal cortical hormones, which is found after whole body x-radiation (Patt, Swift, Tyree, and John, '47; and Patt, Swift, Tyree, and Straube, '48), is the responsible factor for the deposition of liver glycogen in irradiated animals. The fact that the adrenals increased in size even though protected by lead is strongly indicative that the radiation effects on the adrenals are secondary effects. That these effects are secondary is also supported by the findings of Straube, Patt, Tyree, and Smith, ('49), that no significant difference appeared in radiosensitivity between normal and adrenalectomized rats after whole body x-radiation. It may be that substances are liberated from radiosensitive cells, which in addition to giving rise to large deposits of glycogen may also initiate other changes, particularly those (Selye, '49) associated with the adrenal alarm response which lead to increased adrenal hormone secretion and hypertrophy.

It is possible that products of glycogen metabolism in muscle are transferred to the liver and converted to hepatic glycogen, but it is more probable that a generalized increased rate of protein catabolism (Selye, '49) and that the release of substances from radiosensitive cells are responsible (Ely and Ross, '48; and Ross and Ely, '49) for the deposition of hepatic glycogen after radiation.

SUMMARY

Glycogen deposits were found after neutron or x-radiation in the liver cells of rats fasted prior to and after radiation. Glycogen deposits were also found in fasted irradiated rats after shielding of various portions of the rat body. The effect of radiation on glycogen deposition, therefore, is not a primary one.

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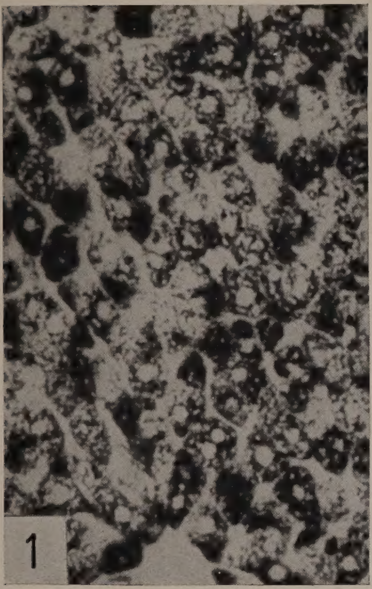
PLATE 1

EXPLANATION OF FIGURES

Figures 1 to 4 Distribution of glycogen in liver sections of rats. Glycogen, demonstrated by periodic acid-leucofuchsin method, appears as black deposits. × 340.

1 Unirradiated, food *ad libitum.*
2 Unirradiated, fasted for 24 hours (print overexposed to show detail).
3 Unirradiated, fasted for 48 hours (print overexposed to show detail).
4 Irradiated (100 n), fasted 24 hours before and 24 hours after radiation.



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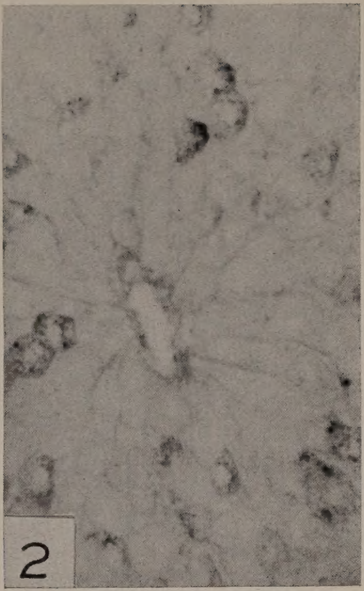

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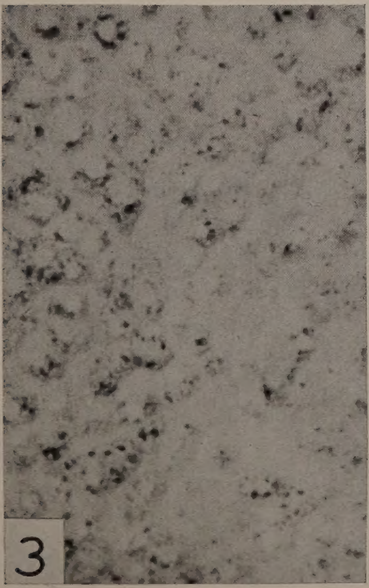

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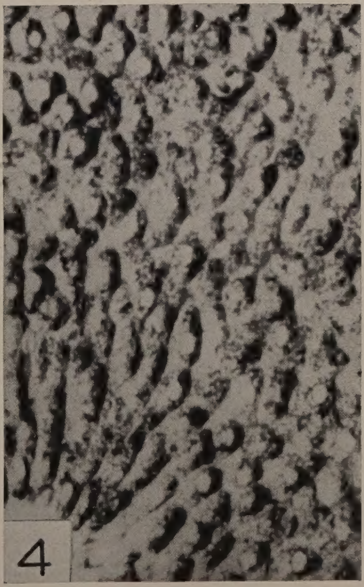

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